Requirement of *MSI1* for Seed and Fruit Development in *Arabidopsis thaliana*

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Olivier LEROY

aus

Frankreich

Promotionskomitee

Prof. Dr. Claudia Köhler (Leitung der Dissertation)

Dr. Lars Hennig

Prof. Dr. Ueli Grossniklaus (Vorsitz)

Dr. Justin Goodrich

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Promotionskomitee:

Prof. Dr. Ueli Grossniklaus (Vorsitz)

Prof. Claudia Köhler (Leitung der Dissertation)

Dr. Lars Hennig

Prof. Enrico Martinoia

ZUSAMMENFASSUNG

Die Proteine der *Polycomb-Gruppe* (PcG) sind evolutionär konservierte Proteine, die die Transkriptionmuster von Zellen über Generationen aufrechterhalten. Der *FERTILIZATION INDEPENDANT SEED* (*FIS*) PcG Komplex von Pflanzen hat eine ähnliche Zusammensetzung wie der „Polycomb Repressive Komplex 2" von Tieren. Mutationen in den Genen der FIS-Gruppe verursachen einen „Parent-of-origin" abhängigen Entwicklungsstopp der Samen. Samen, die ein mutiertes *fis* Allel von der Mutter erben, abortieren unabhängig von der Anwesenheit des väterlichen Wildtyp-Allels. Im *fis*-mutanten Gametophyten durchläuft ausserdem die Zentralzelle auch ohne Befruchtung mehrere Teilungen und es kommt zur Bildung einer endospermähnlichen Struktur.

Ein Vertreter der Gruppe der *FIS* Gene ist *MSI1*. Eine spezifische Eigenschaft der *msi1* Mutante ist die Fähigkeit der Eizelle, parthenogenetische Embryos zu bilden. Um den Mechanismus der FIS-Funktion vor der Befruchtung zu verstehen, suchte ich nach einer Suppressor-Mutante des autonomen Samenentwicklungsphänotyps von *msi1*. Zwei Suppressoren konnten identifiziert werden und wurden als *modifier* (*mod*) bezeichnet. Diese beiden Suppressoren brechen nicht nur nach einigen Teilungen die autonome Samenentwicklung ab, sondern verhindern auch die Bildung des parthenogenetisches Embryos. Nach der Befruchtung brechen *msi1,mod* Samen ihre Entwicklung im globulären Stadium ab, aber das Endosperm entwickelt sich nicht. Die *mod* Mutation alleine verursacht Embryo-Lethalität, wobei der Embryo das Wachstum im präglobulären Stadium beendet. Die *msi1,mod* Doppelmutante dagegen hat mit *msi1* einen synergistischen Effekt auf die Entwicklung des Endosperms, das Wachstum des Endosperms wird nach einigen Zellteilungen angehalten. Beide *mod* Loci kosegregieren mit der *msi1* Mutation und sind mit dem *msi1* Locus gekoppelt (16 cM).

Die *msi1* Mutante bildet zwei Klassen von Samen: Samen, die früh im globulären Stadium, und Samen die später im *fis*-artigen Herzstadium die Entwicklung abbrechen. Ich konnte demonstrieren, dass die früher abortierenden Samen homozygot für die *msi1* Mutation sind, während die später abortierenden Samen heterozygot für die *msi1* Mutation sind und das *msi1* mutierte Allel vom weiblichen Gametophyten erbten. Weiters lieferten meine Studien Beweise, dass - im Gegensatz zu den *FIS* Genen *MEDEA* und *FIS2* - *MSI1* nicht durch Imprinting reguliert wird. Drei Tage nach Befruchtung wird das väterliche *MSI1*

Allel sowohl im Embryo also auch im Endosperm exprimiert. Außerdem konnte ich nachweisen, dass Expression von *MSI1* unmittelbar nach Befruchtung, die *msi1*-mutanten Samen nicht retten konnte. *MSI1* muss daher im weiblichen Gametophyten anwesend sein, um die Entwicklung lebensfähiger Samen sicherzustellen, wodurch *msi1* sich als eine weibliche gametophytische Mutation klassifiziert.

SUMMARY

Polycomb group (PcG) proteins are evolutionary conserved proteins that stably maintain established transcriptional patterns over cell generations. The FERTILIZATION INDEPENDENT SEED (FIS) PcG complex from plants has a similar composition as the Polycomb Repressive Complex 2 (PRC2) from animals. Mutations in *FIS* genes cause parent-of-origin dependent seed abortion. Every seed inheriting a mutant *fis* allele from the mother is going to abort, regardless of the presence of a wild-type paternal allele. Furthermore, without fertilization the central cell of *fis* mutant gametophytes starts to divide and forms an endosperm like structure.

MSI1 belongs to the class of *FIS* group genes. One specific property of the *msi1* mutant is the ability of the egg cell to form parthenogenetic embryos. In order to understand the mechanism of FIS function before fertilization, I searched for suppressor mutants of the *msi1*autonomous seed development phenotype. Two suppressors called *modifiers (mod)* were identified that not only arrested the autonomous endosperm development after a few replication cycles, but also prevented formation of a parthenogenetic embryo. After fertilization, *msi1,mod* seeds arrest development at the globular stage and the endosperm does not develop. The *mod* mutation alone causes embryo lethality with embryo growth arrested at the globular stage. However, the *msi1,mod* double mutant has a synergistic effect with *msi1* on the endosperm development and endosperm growth is arrested after a few cell divisions. Both *mod* cosegregate with the *msi1* mutation and are physically linked to the *msi1* locus (16 cM).

The *msi1* mutant forms two seed classes: seeds aborting early at the globular stage and seeds aborting with a fis-like heart stage embryo. I could show that early aborting seeds are homozygous for the *msi1* mutation, whereas late aborting seeds are heterozygous for the *msi1* mutation and inherited the *msi1* mutant allele from the female gametophyte. My studies provide evidence that in contrast to the *FIS* genes *MEDEA* and *FIS2, MSI1* is not imprinted. The paternal *MSI1* allele is expressed three days after fertilization in the embryo and in the endosperm. Furthermore, I could demonstrate that expression of *MSI1* immediately after fertilization could not rescue *msi1* mutant seeds. Therefore, *MSI1* has to be present in the female gametophyte to assure viable seed development, classifying *msi1* as a female gametophytic mutant.

CHAPTER I GENERAL INTRODUCTION

I.1 Reproduction in *Arabidopsis*

I.1.1 Male and female gametophyte development

The life cycle of flowering plants alternates between a haploid gametophytic phase and a diploid sporophytic phase.

Male and female gametes are formed after meiosis division that is followed by two or three mitotic divisions, respectively (Fig. 1.1 and 1.2). Fusion of male and female gametes gives rise to the zygote that initiates the sporophytic phase. The sporophytic phase encompasses embryogenesis, germination and extends until the plant forms spores in reproductive organs.

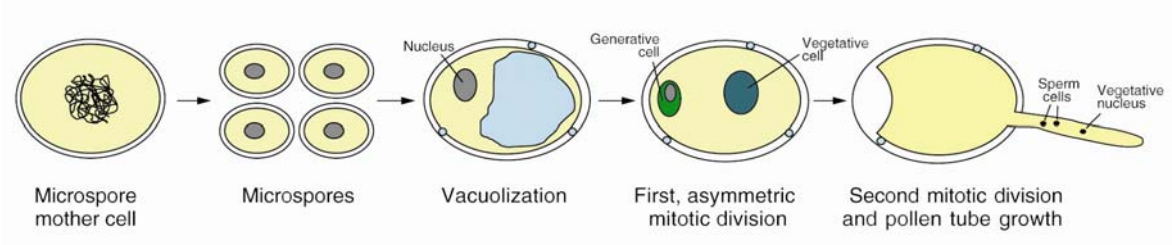

Fig 1.1 Male gametophyte development, from Li and Ma, 2002.

Meiosis produces four microspores. The microspore divides asymmetrically to form a large vegetative cell and a small generative cell. The generative cell produces two sperm cells, which will move towards the ovule through the growing pollen tube.

Both male and female sporocytes (=spore mother cell) undergo a meiosis leading to the formation of four haploid spores, called microspores and megaspores, respectively (Yang and Sundaresan, 2000; Fig 1.1). Within the anther, each microspore matures into a pollen grain. The pollen grain is composed of a large vegetative cell that accumulates reserves, which serve to build pollen tube and two generative cells that comprise the two male gametes involved in the fertilization process

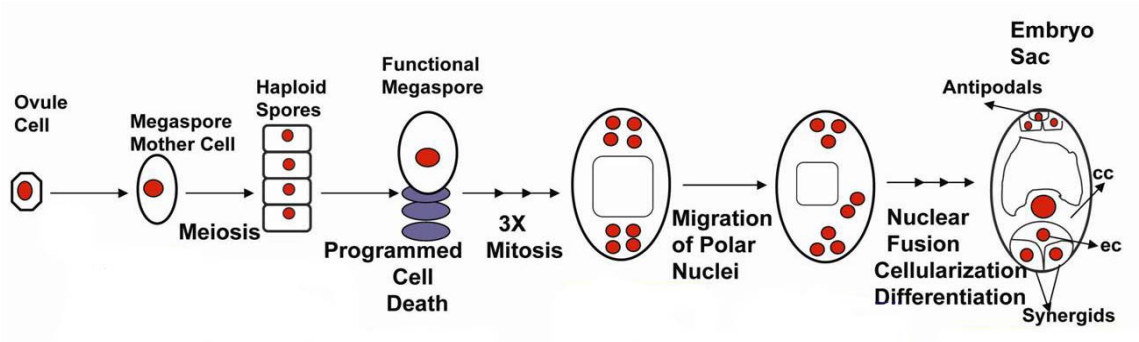

Fig 1.2 Female gametophyte development, adapted from Yadegari and Drews, 2004. Meiosis of the megaspore mother cell produces four haploid spores. Three will degenerate and one will develop into the functional megaspore. During development, three rounds of mitosis form two clusters of four nuclei at the two ends of the female gametophyte. Nuclei migration and cellularization generates seven cells: one egg cell and two synergid cells form the egg apparatus at the micropylar end, and three antipodal cells at the chalazal end. In the large central cell, two nuclei migrate towards the center and fuse together.

After meiosis of the megaspore mother cell, only one of the four megaspores will survive. This cell will undergo three rounds of mitotic divisions giving rise to the seven-celled female gametophyte (Fig 1.2).

The mature female gametophyte in *Arabidopsis* is approximately 105 μm in length and approximately 25 μm in width. At the micropylar end (Fig 1.3), a triad of cells is found: the medial egg cell and two lateral synergids. At the chalazal end three antipodal cell are located. In the center of the female gametophyte is the central cell containing two polar nuclei. Shortly before female gametophyte maturation, two important processes occur: the antipodal cells degenerate and the two nuclei of the central cell fuse. The female gametophyte is surrounded by two sporophytic tissues necessary for its maturation: the internal and external integuments (Yadegari and Drews, 2004; Ray et al., 1996).

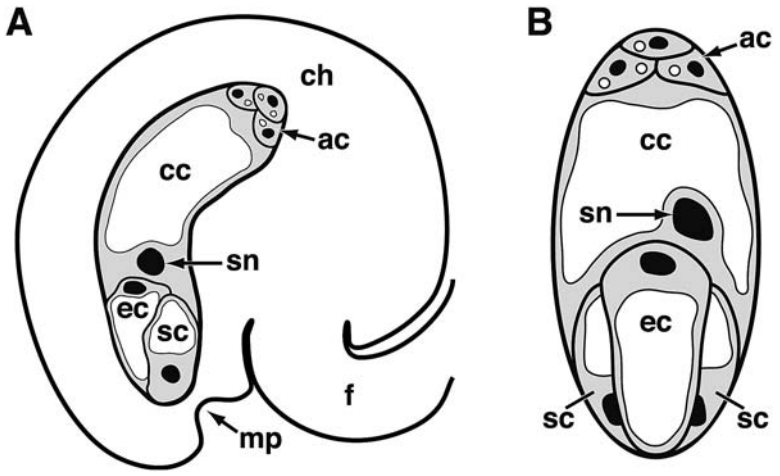

Fig 1.3 Schematic view of the *Arabidopsis* ovule (A) and female gametophyte (B), from Drews and Yadegari, 2002. View in (**B**) is perpendicular to that in (**A**). Within the female gametophyte, the gray areas represent cytoplasm, the white areas vacuoles, and the black areas nuclei. Abbreviations: ac, antipodal cells/ cc, central cell/ch, chalazal region/ ec, egg cell/f, funiculus/ mp, micropyle/sc, synergid cell/ sn, secondary nucleus.

I.1.2 Seed development

The double fertilization process was discovered independently by Navashin and Guignard over a century ago. Fertilization requires that pollen grains germinate on the stigma. After hydration, the vegetative cell forms a pollen tube that grows through the style towards the ovule. The pollen tube enters the female gametophyte through the micropyle (Fig 1.4) and releases the two sperm cells into one of the synergid cells. One of the sperm cell fuses with the haploid egg cell forming a diploid embryo and one sperm cell fuses with the homo-diploid central cell forming the triploid endosperm. The endosperm is a transient tissue, which is almost completely consumed by the embryo during development (Berger, 1999).

Seed development can be divided into three successive phases starting with an early embryogenesis phase, spanning from the one cell zygote reaching to the heart stage. The

early embryogenesis phase is characterized by establishment of the two embryonic axes. Along the apical-basal axis different organs are established and through the radial axis different tissues are outlined (Jürgens et al., 2001). The second phase of seed development, extending to embryo maturation, is characterized by accumulation of reserves within the cotyledons and the hypocotyl and by growth of the established organs. Finally, a drastic reduction of the seed water content characterizes the desiccation phase. Seed desiccation is connected with an increase of the abscisic acid content and establishes a dormancy state that allows the seed to resist physical and chemical stresses (Vicente-Carbajosa and Carbonero, 2005).

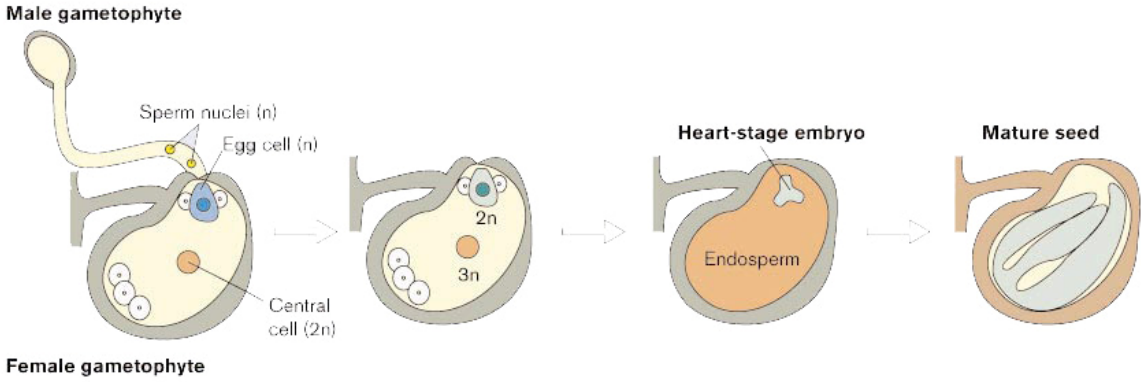

Fig 1.4 Double fertilization and seed development, from Mora-Garcia and Goodrich, 2000. During the fertilization process the two male sperm cells fuse with the haploid egg cell and the diploid central cell, resulting in the formation of a diploid embryo and a triploid endosperm, respectively. As the seed matures, the endosperm is absorbed by the embryo.

I.1.2.1 Embryo development

Embryonic morphology organizes itself around two axes, the apical-basal and the radial axes that are set during early embryogenesis (Jürgens et al., 2001).

The apical-basal axis is composed of different organs: the shoot apical meristem, the cotyledons, the hypocotyl, the radicle and the root meristem. The radial axis corresponds to the tissues organized in concentric layers around the apical-basal axis: the epidermis, the cortex, the endoderm, the pericycle and the vascular tissue are found from the periphery to the center of the radial axis

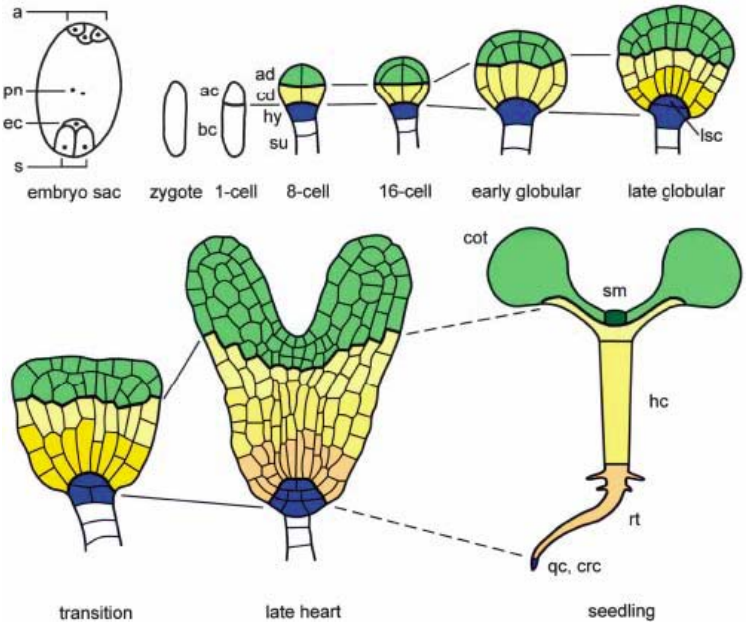

Fig 1.5 Analysis of *Arabidopsis* early embryo development, from Laux et al., 2004. Schematic view of longitudinal median sections of the embryo. The upper and lower thick lines represent clonal boundaries between the descendants of the apical and basal daughter cells of the zygote and between the apical and central embryo domains, respectively. Below each figure the embryo developmental stage is indicated. See text for explanation. Abbreviations: a: antipodes; ac: apical daughter cell ; ad: apical embryo domain; bc: basal daughter cell; cd: central embryo domain; cot: cotyledons; crc: central root cap; ec: egg cell; hc: hypocotyls; hy: hypophysis; lsc: lens-shaped cell; pn: polar nuclei; qc: quiescent center; rt: root; s: synergids; sm: shoot meristem; su: suspensor.

Following fertilization, the zygote elongates and undergoes an asymmetric division establishing a small apical cell and a large basal cell (West and Harada, 1993; Fig 1.5 one-cell stage). The apical cytoplasm-rich cell develops into the embryo proper and the large

vacuolized basal cell develops into the suspensor, composed of six to nine cells. The suspensor fixes the position of the embryo within the seed and supports embryo growth (Yeung and Meinke, 1993). During embryogenesis, the suspensor degenerates except its apical cell that will take part in forming the embryo's root.

In *Arabidopsis*, the small apical cell follows a conserved pattern of cell divisions (West and Harada, 1993). The first two longitudinal divisions followed by a transversal one, lead to the octant stage (Fig 1.6 eight-cell stage). The apical cell row of the embryo will form the cotyledons and the shoot apical meristem, whereas the basal cell row of the embryo develops into hypocotyl and roots meristem. The following periclinal division leads to the sixteen cell stage and initiates radial axis formation. The first embryo tissue is the precursor of the epidermis, namely the protoderm, (Fig 1.5 sixteen-cell stage). Further periclinal divisions give rise to the vascular primordium surrounded by ground tissue: the embryo is then at the globular stage (Laux et al., 2004).

The transition from the globular to the heart stage is defined by the acquisition of a bilateral symmetry. Bilateral symmetry is acquired with the appearance of the two cotyledons developing around the apex, accompanied by the formation of the apical and root meristems. The apical and root meristems are responsible for the plant post embryonic growth and architecture. At heart stage (three to four days after fertilization) the organization plan and the main tissues of the seedling are established (Fig 1.5 heart stage, Jürgens, 2001) .

The last stage in embryogenesis, known as cotyledon stage, is characterized by an active phase of cell division and elongation in which the cotyledons are rapidly growing. This phase is also characterized by an accumulation of proteins and lipid reserves (Vicente-Carbajosa and Carbonero, 2005).

I.1.2.2 Endosperm development

Endosperm development differs dramatically from embryo development (Fig 1.6). In *Arabidopsis* the first divisions of the primary endosperm nucleus are not followed by cytokinesis, giving rise to the formation of a syncytium. Distinct nuclear-cytoplasmic

domains form, the chalazal endosperm at the posterior pole, the micropylar endosperm at the anterior pole and peripheral endosperm domains (Brown et al., 1999; Boisnard-Lorig et al., 2001). Endosperm cellularization is initiated around the globular to early heart stage of embryo development and starts in the micropylar endosperm, which surrounds the embryo, to progress through the peripheral endosperm to the chalazal region (Brown et al., 1999; Boisnard-Lorig et al., 2001). As the embryo matures, most of the endosperm is degraded and absorbed by the embryo, and only a thin aleurone-like layer remains. The endosperm is considered to support embryo growth and to regulate nutrient transfer from the mother to the developing seeds (Lopes and Larkins, 1993).

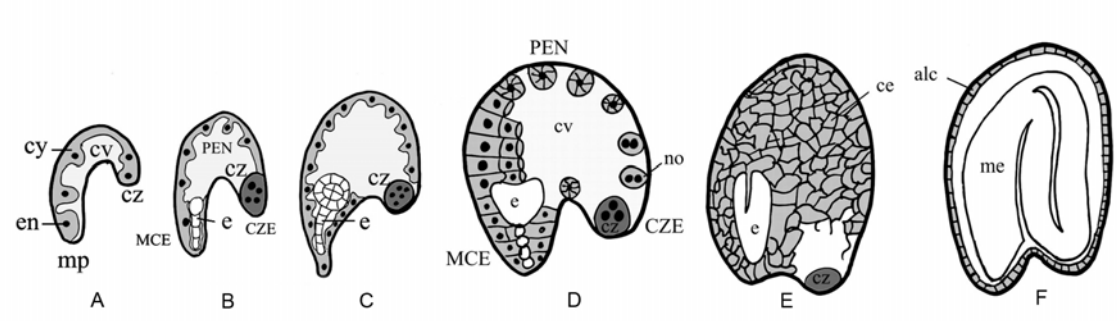

Fig 1.6 Analysis of *Arabidopsis* Endosperm development, adapted from Olsen, 2004. Endosperm development is divided in two major phases, first the Syncytial (**A** to **C**) and then the cellular phase (**D** to **F**). (**A**): After fertilization, the central vacuole (cv) of the central cell enlarges, the cytoplasm (cy) and the nuclei (en) of the endosperm syncytium have a peripheral position. The endosperm divides synchronously and nuclei migrate from the micropylar region (mp) toward the chalazal end (cz); (**B**): As development progresses, the endosperm develops three distinct regions: the region surrounding the embryo (e) (MCE), the central or peripheral endosperm (PEN), and the region of the chalazal endosperm (CZE), which contains the chalazal cyst (cz); (**C**): At the end of the globular embryo stage, the embryo becomes completely surrounded by cytoplasm; (**D**): At the heart embryo stage cellularization of MCE occurs, which is going to propagate to the PEN, while endosperm nodules (no) as well as chalazal cyst (cz) are formated in CZE; (**E**) The endosperm is completely cellularized (ce); (**F**) The endosperm is consumed during seed maturation, leaving only the peripheral aleurone-like cell (alc) layer in a mature embryo (me).

I.2 Epigenetic regulation of seed development

Epigenetics is defined as the study of mitotically and/or meiotically heritable alterations of gene function that do not involve changes in DNA sequence. A classical example of an epigenetic phenomenon in *Arabidopsis* is the vernalization response (Amasino, 2004), during which a transient environmental signal, i.e. cold, leads to a stable epigenetic state which triggers a developmental response, i.e. flowering. The developmental response can occur many months after the initial signal, thus the memory of the initial signal is stably inherited through several mitotic divisions.

I.2.1 Genomic imprinting

Genomic imprinting defines parent-of-origin-specific gene expression: only one of the two inherited alleles is expressed, either the maternally or the paternally transmitted one. Many genes in *Arabidopsis* are exclusively maternally expressed between three to four days after pollination but it is not clear whether they are regulated by imprinting or whether their transcripts are made before fertilization and stored in the female gametes (Vielle-Calzada et al., 2000). The molecular basis for the transcriptional inactivity of the paternal genome is not yet understood. However, this phenomenon is different to genomic imprinting, where biallelic expression of the genes is rarely observed even if in mammals (and maize) it is not uncommon that imprinting is stage- or tissue-dependant. Only a limited number of genes were shown to be imprinted in *Arabidopsis*.

PHERES1 is the first gene that was demonstrated to be maternally repressed and almost exclusively expressed from the paternal allele (Köhler et al., 2005). *PHERES1* is a direct target gene of the FIS (FERTILIZATION INDEPENDENT SEED) *Polycomb* group complex (Köhler et al., 2003a). *PHERES1* belongs to the type I-MADS box transcription factor family and is transiently expressed paternally during early endosperm development. In *fis* mutant seeds, however, *PHERES1* remains overexpressed until the seed aborts.

Genes that have been demonstrated to be imprinted and maternally expressed are *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) and *FWA* (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Jullien et al., 2006, Kinoshita et al., 2004).

I.2.2 *Polycomb* group complexes in animals

Polycomb proteins (PcG) were first identified in *Drosophila* as proteins that maintain a cellular memory of homeotic gene expression (Simon, 1995). They act antagonistically to the *trithorax* group proteins (TrxG), which are either transcriptional coactivators or chromatin remodeling factors (Kennison and Tamkun, 1998). PcG bind and act through DNA sequences called *POLYCOMB RESPONSE ELEMENTS* (*PRE*) and the TrxG bind and act through DNA sequences called *TRITHORAX RESPONSE ELEMENTS* (*TRE*) that are either identical or closely linked to PRE sequences (Orlando et al., 1998; Tillib et al., 1999; Brock and van Lohuizen, 2001; Papp and Müller, 2006). The repression effect of the PcG proteins is antagonized by transcription of non-coding RNAs through the *PRE* that prevents the establishment of silencing (Schmitt et al., 2005). Thus far, *PRE*s have only been characterized in *Drosophila.* There are two distinct classes of PcG complexes: the *Polycomb* Repressive Complex 2 (PRC2) and the PRC1.

In *Drosophila,* the PRC2 contains the core-subunits Enhancer of Zeste (E(z)), Extra Sex Combs (Esc), Supressor of Zeste (Su(z)12) and p55 (Tie et al., *2001;* Birve et al., 2001; Ringrose and Paro, 2004). PRC2 is recruited to *PREs* and trimethylates lysine 27 of histone H3 (H3K27me3). Methylation activity is conferred by the subunit E(z) (Cao and Zhang, 2004) that contains a catalytic "Supressor of variegation, Enhancer of Zeste and Trithorax" (SET) domain (Tschiersch et al, 1994; Rea et al., 2000). E(Z) is enzymatically inactive on its own but depends on the other PRC core subunits for stimulating its enzymatic activity (Schwartz and Pirrotta, 2007). The H3K27me3 domains span up to 100 kb around the originally methylated *PRE* site, suggesting that PRC2 transiently binds to *PRE* surrounding regions (Schwartz et al., 2006). In *Drosophila,* as well as mammals and flowering plants, mutations leading to loss of function of the PRC2 complex result in severe early embryonic phenotypes, implying that PRC2 possesses other functions than regulating homeotic genes. This is supported by genome wide profiling experiments that revealed that PRC2 complexes regulate all major developmental pathways (Ringrose, 2007).

The PRC1 contains the subunits encoded by Polycomb (Pc), Posterior sex comb (Psc), Polyhomeotic (Ph) and Sex comb extra (Sce/RING) (Franke et al., 1992; Satijn et al., 1997; Shao et al., 1999). The chromodomains of the Pc protein can bind to H3K27me3 (Fischle et al., 2003). However, PRC1 binding is found not only at sites containing H3K27me3, suggesting that PRC1 is recruited by H3K27me3-independent signals (Papp and Muller, 2006). The PRC1 complex has also histone modification activity. The RING subunit that has E3 ubiquitin ligase activity and ubiquitinates histone H2A (Wang et al., 2004). PRC1-mediated histone ubiquitination is necessary for gene silencing and plays a role in mammalian X chromosome inactivation (de Napoles et al., 2004).

Recent research revealed a third PRC in *Drosophila*, named Pleiohomeotic Repressive Complex (PhoRC). PhoRC combines DNA-targeting activity (Pho) with a unique methylated-histone-binding activity (dSfmbt). It has been proposed that *PRE*-tethered PhoRC selectively interacts with methylated histones in the chromatin flanking PREs to maintain a *Polycomb*-repressed chromatin state (Klymenko, 2006).

I.2.3 *Polycomb* group complexes in plants

Arabidopsis contains homologs of all major PRC2 subunits and most of them are encoded by more than one gene. The subunits combine into several PRC2 like-complexes that are required for the regulation of various developmental pathways.

Drosophila protein	*Arabidopsis* Protein	Protein domains
E(Z)	MEA, CLF, SWN	SET
ESC	FIE	WD40
SU(Z)12	FIS2, EMF2, VRN2	C2H2 Zn finger
p55	MSI1-5	WD 40

Table 1.1 PRC2 complex subunits in *Drosophila* and *Arabidopsis*

E(Z) homologs in *Arabidopsis* (Table 1.1) constitute a family of three genes: *CURLY LEAF* (*CLF*; Goodrich et al., 1997), its closest relative *SWINGER* (*SWN*; Chanvivattana et al., 2004) and *MEDEA* (*MEA*; Grossniklaus et al., 1998). Three genes homologues to Su(z)12 have been characterized in *Arabidopsis* (Table 1.1): *FERTILISATION INDEPENDENT SEED 2* (*FIS2;* Luo et al., 1999), *EMBRYONIC FLOWER 2* (*EMF2;* Yoshida et al., 2001) and *VERNALISATION2* (*VRN2;* Gendall et al., 2001). There are five A*rabidopsis* homologs to p55: *MSI1* (Köhler et al., 2003b), *MSI4* (Kim et al., 2004), and *MSI2,3,5* that have not yet been functionally characterized. The *ESC* homolog *FERTILIZATION INDEPENDENT ENDOSPERM* is represented by a single gene in *Arabidopsis* (Ohad et al., 1999).

The Su(z)12 homologs FIS2, EMF2 and VRN2 are specific subunits for different PRC2-like complexes in *Arabidopsis*. Thus, FIS2 is a subunit of the FIS complex that acts together with MEA, FIE and MSI1 (Köhler et al., 2003b; Chanvivattana et al., 2004). EMF2 acts together with CLF, FIE and an as yet undefined MSI subunit to represses flowering transition (Chavivattana et al., 2004). Finally, VRN2 acts together with CLF or SWN, FIE and an as yet undefined MSI subunit to mediate the vernalization response (Wood et al., 2006).

Studies accumulate and establish that in *Arabidopsis* the attributed PRC2 functions are more intricate than previously thought. Thus, different complexes regulate common sets of target genes during the different stages of plant development (Makarevich et al., 2006). Furthermore, different PcG genes act redundantly, like *CLF* and *SWN* (Chanvivattana et al., 2004) as well as *MEA* and *SWN* (Wang et al., 2006).

Plants do not possess a canonical PRC1 complex. However, the LIKE HETEROCHROMATIN PROTEIN1 (LHP1) protein was recently shown to bind to H3K27me3. H3K27me3 binding sites clearly overlap with binding sites for LHP1, suggesting that LHP1 has a similar functional role like the PRC1 complex in animals (Turck et al., 2007). Nevertheless, the phenotypes of PcG mutants and the *lhp1* mutant do not completely overlap, making it likely that additional mechanisms are needed to establish PcG-mediated gene repression. Thus, the distribution of the H3K27me3 mark in

Arabidopsis is mainly found in genes at the 5'-end of the transcribed region, suggesting that PRC2 interacts with the transcription apparatus bypassing the need of the activity attributed to the PRC1 complex (Zhang et al., 2007).

I.2.3.1 The FIS complex

The *fis* mutants have been identified in a screen for mutants that can form seeds in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997). The *FIS* group genes identified in this screen are *FIS1* (*MEA), FIS2 and FIS3 (FIE). MEA* was also independently identified as a gametophytic maternal effect mutant (Grossniklaus et al., 1998). *MSI1* was identified by a reverse genetics approach based on homology to the p55 subunit from Drosophila (Köhler et al., 2003b) as well as in a forward genetic screen (Guitton et al., 2004). All four genes are expressed in the female gametophyte before fertilization and in the endosperm after fertilization (Grossniklaus et al., 1998; Luo et al., 2000; Yadegari et al., 2000, Köhler et al., 2003b). However, *FIE* and *MSI1* are also expressed during sporophytic development, corresponding with their roles in different PRC2-like complexes (Yadegari et al., 2000; Köhler et al., 2003b). *MEA* and *FIS2* show imprinted expression in the endosperm, with only the maternal allele being expressed and the paternal allele remaining silent (Kinoshita and al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Jullien et al., 2006).

All *fis* mutants are characterized by autonomous seed formation in the absence of fertilization (Chaudhury et al., 1997; Ohad et al., 1996; Kohler et al., 2003b; Guitton et al., 2004). *fis* mutant female gametophytes are relieved from a repressive block in the central cell, causing the central cell nucleus to divide resulting in the formation of a diploid endosperm. In contrast to other *fis* mutants, *msi1* mutant gametophytes also form parthenogenetic embryos at a relatively high frequency (Guitton and Berger, 2005). The autonomously growing egg cell in *msi1* gametophytes elongates and divides asymmetrically, similar to a developing fertilized egg cell. The *msi1* parthenogenetic haploid embryo can reach up to 20 cells and expresses embryo markers. Both, the diploid *msi1* central cell and the haploid *msi1* egg cell acquire the ability to develop autonomously.

Therefore, *MSI1* plays an essential role in repressing central cell and egg cell development in the absence of fertilization.

When the *fis* ovules become fertilized, embryo growth is arrested at heart stage and the endosperm fails to cellularize and overproliferates (Grossniklaus et al., 1998; Kiyosue et al., 1999). *MEA* and *FIS2* are paternally expressed imprinted genes, and *FIE* is paternally silent during early stages of endosperm development but it is not known whether it is regulated by imprinting or not (Vielle-Calzada et al., 1999; Jullien et al., 2006; Yadegari et al., 2000). Therefore, a *mea, fis2 or fie* female gametophyte fertilized by wild-type (wt) pollen leads to fertilization products in which *MEA, FIS2* and *FIE* are not expressed, respectively. This lack of paternal *FIS* gene expression could explain why *fis* mutants fail to be rescued by a wild-type paternal *FIS* allele.

I.2.3.2 MSI1 is a subunit of different complexes

Similar to *mea*, *fis2* and *fie* mutants, lack of MSI1 function causes parent-of-origin-dependent seed abortion. However, it has been proposed that lack of MSI1 function causes in addition to the gametophytic effect also a sporophytic effect on seed development (Guitton et al., 2004). Thus, lack of both, maternal and paternal *MSI1* alleles causes a significant stronger defect than lack of the maternal *MSI1* allele alone. This implies that the paternal allele of *MSI1* is active, but fails to complement the maternal gametophytic *msi1* defect. The strong sporophytic defect of *msi1* mutant embryos could be explained by multiple functions of MSI1 during plant development. MSI1 is a subunit of several other PRC2-independent complexes. Thus, MSI1 binds to the retinoblastoma protein (Ach et al., 1997). MSI1 is also part of the Chromain Assemby Factor 1 (CAF-1) complex together with FASCIATA 1 (FAS1) and FASCIATA 2 (FAS2) proteins (Kaya et al., 2001). The CAF-1 complex plays a role in establishing an ordered nucleosome structure by facilitating deposition of nucleosomes on newly synthesized DNA. The CAF-1 complex binds to newly synthesized histone H3 and (acetylated) H4 and mediates the formation of the H3-H4 tetramer on newly replicated DNA (Mello and Almouzni, 2001). The CAF-1 complex

maintains the activity of both, root and shoot apical meristems by restricting *WUSCHEL* and *SCARECREW* expression (Kaya et al., 2001).

I.3 Aim of this thesis

The aim of my thesis was to identify suppressor mutants of the *msi1* autonomous seed development phenotype that is caused by a lack of a functional FIS complex. Suppressor mutants could be defective in FIS target genes or in genes counteracting FIS function. Therefore, suppressors of the *msi1* mutant phenotype could help to reveal which genes play important roles during early endosperm development and need to be expressed in order for the fis development to occur. It can be furthermore anticipated that mutants in activating complexes that counteract PcG activities could suppress the *msi1* mutant phenotype.

The second aim of my thesis was to understand the parent-of-origin-dependent seed abortion phenotype of the *msi1* mutant. I aimed to address the following questions: i) Why does *msi1* form two different seed classes? ii) Is *MSI1* imprinted, similar to *MEA* and *FIS2*? iii) Can the *msi1* mutant phenotype be rescued by early paternal *MSI1* expression? iv) What causes the *msi1* embryo to abort? Addressing these questions could help to understand whether the parent-of-origin effect of the *msi1* mutant is caused by a defect in the female gametophyte or whether genomic imprinting impacts on *msi1* seed development.

CHAPTER II: MATERIAL AND METHODS

II.1 Plant material

II.1.1 Plant material and growth conditions

Wild-type accession of *Arabidopsis thaliana* refers to the Columbia accession unless stated otherwise.

The *msi1* mutant used in this study was the *msi1-1* allele described by Köhler et al., 2003b. The *mea* mutant used in this study was the *mea-1* allele described by Grossniklaus et al., 1998. The *fis2* mutant used in this study was the *fis2-1* allele described by Luo et al., 1999.

The silent *MSI1** allele, which encodes a wild-type MSI1 protein, is a TILLING (Induced Local Lesions IN Genomes) mutant obtained from the Nottingham Arabidopsis Stock Centre, stock number CS92951.

The *delayed-dehiscence2 (dde2)* mutant was initially described by von Malek, et al., 2002. The *dde2-2* allele was obtained from the Nottingham Arabidopsis Stock Centre, stock number 2379.

The *35S::GUS* line was kindly provided by Célia Baroux.

The *PHEI::GUS* line was already described by Köhler and colleagues (Köhler et al., 2003a)

The *35S::MSI1* and *PHEI::MSI1* plasmids were kindly provided by Lars Hennig.

Used marker lines are specified in table 2.1.

Marker for	Construct	Selection	Reference
Auxin	*DR5::GFP*	Sulphonamide R	Friml et al., 2003
Vascular tissue	*Gal4::GFP*	Kanamycine	N9217
Quiescent center	*SCR::YFP*	Basta®	Wysocka-Diller et al., 2000
Basal cell lineage	*Wox8::vYFP*	Basta®	Laux et al., 2004
Shoot apical meristem	*Gal4::GFP*	Kanamycine	N9335
	Gal4::GFP	Kanamycine	N9336
Constitutively expressed gene	*35S::GUS*	Hygromycine	Provided by Celia Baroux
FIS target gene	*PHE::GUS*	Hygromycine	Köhler et al., 2003a
Female gametophyte	*DD46::GUS*	Hygromycine	This work

Table 2.1 Marker lines used for this study.

Seeds were surface sterilized with bleaching solution (5 % sodium hypochlorite and 0.01 % Tween 20) for 10 min at room temperature, washed with sterile deionized water and plated on sterile 0.8% agar MS plates (Murashige and Skoog, 1962).

According to the selection gene carried by the transgene, we added different selective agent to the MS plates (Table 2.2)

Resistance gene	Selective agent	Final concentration
Bar	Phosphinothricine	30 mg/L
nptII	Kanamycine	25 mg/L
Hyg	Hygromycine	25 mg/L

Table 2.2 Selective agents used in MS medium

Seedlings were grown on MS medium with daily cycles of 16 h light phase and 8h dark phase at 22°C. After 10 days seedlings were transferred to soil in a greenhouse facility with 70% relative humidity and a daily cycle of 16 h light phase at 21°C and 8 h dark phase at 18°C.

Anthers about to shed their pollen were removed from flowers (emasculated). After 24 hours pollen was applied to the stigma of the emasculated flower, referred to as day zero after pollination (DAP0).

fis mutants were immediately pollinated after emasculation in order to prevent autonomous seed formation.

II.1.2 Generation of transgenic lines

Twelve to fifteen flowering plants were dipped in *Agrobacterium tumefaciens* suspension for 10 sec. The plants were covered till the next day in order to keep a high degree of humidity. They were not watered for the next 2 days and grown 3 weeks allowing the transformed seeds to mature. T1 transformants were selected on MS plates (Murashige and Skoog, 1962) containing the required antibiotics.

All transgenic lines established during this work are indicated in table 2.3. The construction of the vectors is indicated in paragraph II.4.4.

Promoter	Construct	Plant selection
MSI1	*MSI1::MSI1-GFP*	Hygromycine
PHERES1	*PHE1::MSI1*	Hygromycine
	PHE1::FIS2	Kanamycine
DD46	*DD46::MSI1*	Hygromycine
	DD46:: GUS	Kanamycine

Table 2.3 Transgenic lines made during this study.

II.2 Plant phenotypic analysis

II.2.1 Seed preparations for histology

Phenotypic characterization of seeds was performed as follows: siliques were fixed in ethanol:acetic acid (9:1) for 24 h at 4°C. Then they were successively washed in 90%, 80%, and 70% ethanol for 1h each at 4°C. Finally, they were cleared for 24 h in chloral hydrate solution (80 g chloral hydrate (Fluka), 20 g glycerol (Sigma) and 10 mL distilled water). Siliques were dissected with insulin injection needles (Becton Dickinson), mounted on glass slides and observed under a microscope DM2500 (Leica) with Normaski filter. Pictures were taken with a CCD camera DFC300FX (Leica).

II.2.2 Seed GUS staining

GUS assays were performed as described below:

Each silique valve was longitudinally incised with insulin injection needles and fixed for 1 h in 90% acetone at -20°C. They were washed twice for 15 minutes wih the washing buffer (50 mM Na-phosphate pH, 7.0, 10 mM EDTA, 0.1 % Triton X-100). Siliques were vacuum infiltrated for 20 min in GUS-staining buffer (50 mM Na-phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 1.5 mg/mL 5-bromo-4-chloro-3-indolyl-D-glucuronide salt (Biosynth)). Following vacuum infiltration the siliques were incubated at 37°C in the dark for 24 h. Dissected siliques were cleared in chloral hydrate solution prior to examination using a Leica microscope DM2500 equipped with Nomarski optics. Pictures were taken with the CCD camera DFC300FX (Leica).

II.2.3 Seed/embryo fluorescence assay

Siliques of the plants containing the construct of interest were freshly detached from the branches, dissected in deionized water with insulin injection needles and mounted on glass slides. For embryo observation, a slight pressure over the cover slip with a pencil

removed embryos from the seeds. Samples were examined using a Leica DM2500 equipped with an external fluorescence excitation light source EL6000. The UV filter set used was L5 (excitation filter passant band at 480/40 nm, dichroic mirror at 505 nm, suppression filter at 527/30 nm). Pictures were taken using the Leica CCD camera DFC300FX.

II.3 Genetic screen

We introduced the *dde2* mutation into the *msi1* mutant background by crossing. The *dde2* mutation induces a defect in the jasmonic acid biosynthesis pathway that prevents pollen release for the anther. The *dde2* mutation can be complemented by spraying methyl jasmonate.

Seeds of the genotype *msi1/MSI1, dde2/dde2* were harvested and irradiated in the "Laboratoire de Radiobiologie végétale" in the CEA of Cadarache (France). A Cobalt-60 source was used for γ-irradiation at an intensity of 200 Gray at a rate of 27 Gray/minute.

msi1 M1 plants were treated with methyl jasmonate in order to release pollen and allowed to self fertilize. Two siliques were harvested per M1 plant in individual bags and seeds from one of these siliques was sawn on soil in one pot and selected for the *msi1* mutation.

The M2 families were consecutively checked for silique size variation within each family. Candidates were backcrossed with *msi1/MSI1, dde2/dde2* plants in both directions as well as self fertilized.

II.4 Molecular protocols

All standard molecular biology procedures (e.g. restriction enzyme digestions, ligation reactions or plasmid DNA-preparations) were performed as described in Sambrook et al. (1989) or according to the suppliers' instructions. Restriction endonucleases, DNA modifying enzymes and reaction buffers were purchased from New England Biolabs. *Taq*-DNA polymerase was homemade by Maria Misteli according the protocol of Desai and Pfaffle (1995). For standard molecular procedures, the following kits were used:

GFX® PCR DNA and Gel Band Purification Kit (Amersham)
*p*Drive® & T-A cloning based PCR cloning kit (Qiagen)
SuperScript®-First-Strand Synthesis System for RT-PCR Kit (Life technologies)
Expand® Long Template PCR System (Roche).

II.4.1 PCR reaction

Oligonucleotides were ordered from Sigma and the standard PCR buffer was composed of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, BSA 100 µg/ml, 200 µM dNTP and 200 nM of each oligonucleotide. Standard PCR reactions were conducted in a thermocycler DNA engine DYAD (MJ Research) as follows :

The first step was a DNA melting phase
94°C for 2 minutes
Then 30 to 41 cycles of
94°C for 10 sec for DNA melting
55°C for 30 sec for DNA annealing
72°C for a minimum of 45 sec for amplification, with 1 minute for each kb of amplicon

Then, a final elongation phase of 7 min at 72°C.

II.4.2 Plasmid manipulation in bacteria

Escherichia coli strain TOP10 (Invitrogen):Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L(Str^R) *nup*G was used to propagate plasmid constructs. The cells were treated as described by Hanahan (1983) to make them competent to receive plasmids. Plasmid amplification was achieved by growing transformed bacteria in LB medium (10 g/L yeast extract, 10 g/L tryptone-peptone, 5 g/L NaCl, pH 7.0) supplemented with the antibiotic for which the plasmid harbored the resistance gene (Table 2.5). Plasmid extraction was initiated by a standard alkaline lysis.

Resistance gene	Antibiotic	Final concentration
nptII	Kanamycin	100 mg/L
Hyg	Hygromycin	50 mg/L

Table 2.4 Antibiotics used in LB medium for plasmid selection.

The *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pMP90 (Simoens et al., 1986) was used for plant transformations. The freeze-thaw method was used to generate competent and transformed cells as described below.

Agrobacterium tumefaciens cells were grown overnight at 28°C in 25 mL LB-medium containing 40 mg/L gentamicine and 100 mg/L rifampicine. Bacteria were harvested by centrifugation (10 minutes at 4000 rcf) and resuspended in 1 mL ice-cold 20 mM $CaCl_2$. 100 µL aliquots were shock-frozen in liquid nitrogen.

One µg of purified plasmid DNA was added to the frozen cells, which were subsequently thawed for 5 minutes at 37°C. The cells were incubated in 1 mL LB medium at 28°C for 2 hours and spread on LB plates containing 40 mg/L gentamicine, 100 mg/L rifampicine and the appropriate antibiotic for the prokaryote selection marker carried by the binary vector. After incubation at 28°C for 2 days, a single colony was picked and grown in

300 µL of selective medium. Cells were pelleted for 10 min at 5000 rcf and resuspended in 500 mL of transformation medium (4.6 g/L MS-base salt (Carolina Biological Supplies), 50 g/L sucrose and 0.2 mL/L Silwet-77 (Lehle Seeds) which represented the inoculum for plant transformation.

II.4.3 DNA/RNA manipulation in plants

II.4.3.1 Genomic DNA isolation

For standard PCR reactions, genomic DNA was isolated as follows: single leaves were collected in 1.5 mL Microfuge tubes containing 5-10 glass beads (1.7-2.0 mm in diameter) and immediately frozen in liquid nitrogen. The samples were ground for 6 seconds in a Silamat 7 mixer (Ivoclar Vivadent). DNA extraction was performed with 500 µL Edwards buffer (200 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM EDTA and 0.4% SDS). After centrifugation at 12,500 rcf for 8 minutes, 400 µL of the supernatant was transferred to fresh reaction tubes containing 400 µL isopropanol, then centrifuged as mentioned above and washed with 70% ethanol, dried and redissolved in 50 µL TE (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8).

II.4.3.2 Mutant genotyping

The combinations of primers used for genotyping are listed below (Table 2.5). For genotyping of *FIS2* and *fis2-1* the amplified product had to be digested with *Bsu*36I to differentiate both alleles: the wt *FIS2* allele gave three DNA fragments of about 200 bp whereas the mutant allele one DNA fragments of about 200 bp and one of about 400 bp.

genotype	Primers	size
MSI1	LH135 GATTCTAGGGTTATAACGAGG LH136 GATGCCATGCAACATCTTCCAC	400bp
msi1-1	LB3 TAGCATCTGAATTTCATAACCAATCTCGATACAC LH136 GATGCCATGCAACATCTTCCAC	400bp
DDE2-2	CK326 GAGTCTCCGTCTCCGGTCCAT CK327 TCCATCGGAGCCTAAACACGA	900pb
dde2-2	CK326 GAGTCTCCGTCTCCGGTCCAT CK214 GCGTGGACCGCTTGCTGCAACT	500bp
MSI1	OL66 GTAATCGAAAACATAGACCTCC OL70 CGGTAAAGACTACTCCGTTCAGATG	400bp
*MSI1**	OL66 GTAATCGAAAACATAGA OL69 CGGTAAAGACTACTCCGTTCAGATA	400bp
FIS2 *fis2-1*	OL29 ATGATGAAAATGTATCATCGACACCAAG OL32 ACCGCTCTGCATGTAACTCTTTTCT	600bp

Table 2.5 List of primers used for plant genotyping

II.4.3.3 RNA isolation and reverse transcription-PCR (RT-PCR) analysis

Three flowers or siliques were collected in 1.5 mL microfuge tubes containing 5-10 glass beads (1.7-2.0 mm in diameter) and immediately frozen in liquid nitrogen. For studies involving dissection of embryo from the seeds, samples were kept in 25 µL of RNAlater (Ambion) until all dissections were performed and then frozen in liquid nitrogen. Samples were ground three times for 6 sec in a Silamat 7 mixer (Ivoclar Vivadent) and kept in liquid nitrogen in between. Ground tissues were resuspended in 1 mL Trizol® (Life technologies) and centrifuged for 10 min at 4°C and at 12000 rcf. Samples were incubated for 5 min at room temperature, subsequently, 200 µL chloroform were added; samples were mixed and

incubated for 5 min at room temperature. A 15 min centrifugation at 4°C and at 12000 rcf, resulted in the isolation of the aqueous phase (ca. 600 μL) which was transferred to fresh reaction tubes. The RNA was precipitated with the addition of 0.5 mL isopropanol, washed with 75% ethanol and redissolved in 50 μL distilled water.

For detection of transcripts by RT-PCR, 20 μL of the isolated RNA was treated with 5 units of DNAse I for 1 hour at 37°C. Following purification with phenol:chloroform (1:1) , the RNA was coprecipitated with 20 μg glycogen (Roche Diagnostics) as carrier. The RNA was pre-incubated with oligo(dT) primers at 70°C for 10 minutes and immediately used as template for first strand cDNA synthesis using 200 units of Superscript® reverse transcriptase (Gibco-Life technologies). The cDNA was ethanol precipitated and resuspended in 50 μL distilled water.

For assessing the RNA quality (i.e. no genomic DNA contamination and no RNA degradation), control primers for the *ACTIN3* gene were designed to flank an intron. After amplifications, any genomic DNA contamination would result to an amplification product of higher molecular size than the one coming from the cDNA. The expression of the studied genes or transgenes was assessed with specific sets of primers listed below (Table 2.6)

gene	Primers name and sequence	Amplicon size from genomic DNA	Amplicon size from cDNA
Control *ACTIN3* (At3g12110)	GM50 AACTTTCAACACTCCTGCCATG GM49 CTGCAAGGTCCAAACGCAGA	300 bp	200 bp
All *MSI1* transgenic constructs	MSI995F GCACCGCTCTTCACACATTG OL65 TGGTCACCTGTAATTCACACG	400 bp	400 bp
wt *MSI1*	as1 GTAATCGAAAACATAGACCTCC s1 CGGTAAAGACTACTCCGTTCAGATG	400 bp	300 bp
polymorphic *MSI1**	as1 GTAATCGAAAACATAGACCTCC s2 CGGTAAAGACTACTCCGTTCAGATA	400 bp	300 bp
All *FIS2* transgenic constructs	OL82 GGCGGTAAGGATCTGAGCTAC OL83 TACGAAAACAAAGGGTGATCG	400 bp	400 bp

Table 2.6 Primer pairs used to assess expression of specific gene or transgene.

II.4.4 Vector construction

Promoters of interest were amplified from Columbia genomic DNA. The coding region of the *MSI1* gene was amplified from Columbia cDNA, the coding region of the *FIS2* gene was amplified from Landsberg *erecta* cDNA. Cloning strategies and primers are listed in tables 2.7 and 2.8, respectively. PCR products were cloned into the TA-cloning based *p*Drive vector (Qiagen) and verified by sequencing.

	Reporter *GUS*	*MSI1* complementation	*FIS2* complementation
original plasmid	*p*CAMBIA 1391Z	*p*CAMBIA 1380	*p*CAMBIA 2300
vector modification	n.a.	*MSI1* cDNA & *NOS* terminator	*MYC-FIS2* cDNA & *OCS* terminator
primers used to amplify the cDNA coding sequence	n.a.	*MSI1* cDNA up & *MSI1* cDNA down	*FIS2* cDNA up & *FIS2* cDNA down
Aim	*DD46::GUS*	*DD46::MSI1*	n.a.
primers used to amplify the promoter *DD46*	*DD46* up and *DD46* down		
Aim	*PHE1::GUS*	*PHE1::MSI1*	*PHE1:: FIS2*
primers used to amplify the promoter *PHE1*	*PHE1* up and *PHE1* down		
Aim	n.a.	*MSI1::MSI1-GFP*	n.a.
primers used to amplify the promoter *MSI1*		*MSI1* up & *MSI1* down	
primers used to amplify the *GFP* coding sequence		*GFP* up & *GFP* down	

Table 2.7 Cloning strategies. n.a.: not applicable.

Primer name	Sequence	Cloning site
DD46 up	GTGAATTCGACCACAATAAGTGTAATGC	*EcoR*I
DD46 down	AACCATGGTAAAATCGCCGTTTACAAA	*Nco*I
FIS2 cDNA up	CTCGAGAAATCGATCACACTAAAAGCTGAAGTAGTGG	*Xho*I
FIS2 cDNA down	TCTAGATTAAGATCTTTCATCAACTTCCATAGATTG	*Xba*I
MSI1 cDNA up	GACCATGGGGAAAGACGAAGAGGAAATG	*Nco*I
*MSI1*cDNA down	CGAGATCTCTAAGAAGCTTTTGATGGTTC	*Bgl*II
PHE1 up	CCGAATTCGACTTTAAAATAGTAGAAAAGCTTG	*EcoR*I
PHE1 down	AATTCCATGGATCTCTTATCTTTTTCTTTTGTG	*Nco*I
MSI1 up	CGGGATCCAGGTTTGGAATCGACCAAGA	*BamH*I
MSI1 down	GGACTAGTCATGTCGACCGATGTCTTTGTTATTCCCG	*Spe*I
GFP up	CCATGGTGTGAGCAAGGGCGAGGAG	*Nco*I
GFP down	ACTAGTTTACTTGTACAGCTCGTC	*Spe*I

Table 2.8 Primers used for amplification of fragments of interest

CHAPTER III *msi1* AS DISSECTION TOOL OF SEED AND FRUIT DEVELOPMENT

III.1 Identifying regulators of seed and fruit development

III.1.1 Introduction

Reproduction in flowering plants spans from flower initiation to the release of the mature seeds. Seed development follows fertilization and occurs simultaneously with fruit growth. In *Arabidopsis*, the fertilized ovules develop into seeds, whereas the carpel differentiates and turns into the fruit (Giovannoni, 2004). Fruit development also depends on the specific abscission of floral organs and without fertilization the entire flower senesces (Vivian-Smith et al., 2001). Fruit and seed development are actively repressed before fertilization. However, fruit and seed development are naturally uncoupled from fertilization in plants that undergo parthenocarpy and apomixes, respectively.

In parthenocarpic species, fruits form in the absence of fertilization and the unfertilized ovules senesce (Gorguet et al, 2005). Parthenocarpy is explained as a consequence of an elevated concentration of phyto-hormones (gibberellins) in the ovary, a process that can be artificially induced in different species by exogenous application of phyto-hormones to flowers (Vivian-Smith and Koltunow, 1999). In apomictic species seeds and fruits can be produced without fertilization (Koltunow and Grossniklaus, 2003) even if most apomicts do need fertilization for seed and fruit development (pseudogamous) and only the embryo develop without fertilization). Thus far, understanding of apomixis is mainly descriptive, and the underlying molecular mechanisms still need to be discovered (Bicknell and Koltunow, 2004).

The *fis* mutants in *Arabidopsis* form fruit- and seed-like structures independently of fertilization (Ohad et al., 1996; Chaudhury et al., 1997). In a *fis* mutant ovule, the repressive state instituted by the FIS complex is released and one or more of the derepressed gene(s)

induce(s) endosperm growth. By specifically searching for suppressors of the fis phenotype, I anticipated to identify downstream target genes of the FIS complex and to uncover the molecular events controlling the initiation of endosperm development. I also expected to identify regulators that coordinate growth of seed, endosperm, and seed coat. Thus, the aim of this screen was to deepen our understanding of the coordinated development of seed and fruit induced by signal transduction pathways.

Both seed and fruit development are triggered in a *fis* mutant background. Therefore, fis development could share, if not all, at least one seed/fruit activating pathway of normal seed development. If both developmental processes use the same mechanism, there is the possibility that a suppressor of the fis phenotype could affect seed viability. It is to expect that deleting a master gene for seed enlargement would not only suppress the fis phenotype, but would also severely disturb seed viability. Similarly, affecting a signaling pathway communicating between seed and fruit would prevent the silique to elongate: this lack of elongation would not only constrain the autonomous growth of the *fis* ovule, but would also potentially affect seed growth and viability.

III.1.2 Main tool: *msi1-1*

The *msi1* mutant is a member of the *fis* mutant class. The *msi1* mutant has the highest penetrance of the autonomous seed development phenotype, with almost every gametophyte inheriting an *msi1* mutant allele undergoing fis development (Köhler et al., 2003b). Autonomous endosperm development triggers integument development, thus turning the ovule into a seed-like structure. The growth of the seed-like structure triggers silique expansion, valve maturation and seed shedding (Vivian-Smith et al., 2001).

As well as each fertilized seed influences silique growth in an additive manner, each female gametophyte undergoing fis development is also affecting additively silique growth (Cox and Swain, 2006). Therefore, in an *msi1* background the high frequency fis development is causing strong silique elongation. A suppressor of the fis phenotype can be identified by searching for reduced number of ovules undergoing fis development.

Alternatively, a suppressor of autonomous seed development could be directly assessed by searching for reduced silique elongation from emasculated *msi1* plants.

Selecting by silique size would not only permit to find mutations impairing autonomous endosperm development, it can also provide information about fruit development by affecting carpel expansion in response to the growing seed. Silique size could be affected when signaling pathways between the growing seed and the carpel are impaired. Screening for regulators of seed and fruit development could therefore be efficiently achieved by searching for *msi1* plants impaired in silique elongation after emasculation. In order to recover also recessive sporophytic mutations, the screen was performed in the M2 generation.

III.1.3 Accessory tool: the male conditional mutant *dde2*

I planned to analyze at least one thousand M2 families with at least eight plants per family to have a high probability of obtaining homozygous suppressor mutants. As it would be highly labor intensive and error-prone to screen a large number of plants by hand-made emasculation, I searched for a conditional male sterile mutant that would facilitate the screening process. I decided to use the conditional male sterile *dde2* mutant (von Malek, et al. 2002). The *dde2* mutation affects the *ALLENE OXIDE SYNTHASE* (*AOS*) gene which encodes one of the key enzymes of jasmonic acid biosynthesis. A homozygous *dde2-2* mutant fails to undergo anther dehiscence and the pollen is not released. This phenotype can be complemented by spraying the flower with synthetic methyljasmonic acid, the anthers dehisce and the pollen is released.

III.2 Results

III.2.1 Characterization of the *msi1/MSI1; dde2/dde2* double mutant

In fertilized wild-type and *msi1/MSI1* plants silique length was about 18 mm. In a *dde2* or wild-type background the siliques only slightly elongate from 3 mm to 4 mm. In an *msi1/MSI1; dde2/dde2* background, almost all *msi1* mutant ovules start to develop and trigger silique elongation up to 10 mm. This silique size is comparable to the size of siliques formed after emasculation of *msi1/MSI1* flowers (Fig 3.2 and Fig 3.5; Köhler et al., 2003b). Thus, the *dde2* mutation does not interfere with the ability of *msi1* to undergo autonomous seed development and is, therefore, a suitable tool for this screen.

III.2.2 Plants in M1 generation

Approximately 5000 gamma ray mutagenized *msi1/MSI1; dde2/dde2* seeds were sown directly on soil. After selection for the *msi1* mutant plants, fertilization was induced by spraying methyl jasmonate. The mutagenesis efficiency was estimated by determining the number of embryo lethal (3%) and semi-sterile plants (8%) among 57 M1 plants. This frequency is slightly lower compared to published mutagenesis frequencies obtained using the same intensity of γ-irradiation (Guitton et al., 2004). However, as the ratio was determined in an *msi1* background, it is possible that the *msi1* phenotype masked several embryo lethal phenotypes.

III.2.3 Families screened in M2 generation

Out of 1314 M2 families, 704 M2 families had at least one member carrying the *msi1* mutant allele. Thirty families were identified in which at least one member showed a silique size reduction (Table 3.1). Those families where treated with jasmonic acid to set seeds, and backcrossed to *msi1/MSI1, dde2/dde2* plants. Four families (107, 254, 461 and 478) could not be rescued as each M2 family had only *msi1* plant with a strong reduction in silique size. They could neither set seeds, nor transmit the *msi1* mutation paternally when crossed to a wild-type plant. Therefore, they do not appear in the Table 3.1 below.

Family ID	M2 family size	Plants with reduced silique length in M2
5	3	1
16	7	5
24	4	4
41	7	7
43	1	1
60	6	2
73	1	1
92	2	1
93	4	1
105	2	1
142	3	3
187	8	1
207	1	1
269	2	1
289	2	2
440	3	1
446	4	1
540	6	1
550	30	9
570	1	1
647	1	1
668	4	1
693	10	4
938	3	3
952	2	2
1076	2	2

Table 3.1 M2 mutant lines showing silique length reduction. The line referring to mutant lines confirmed in the M3 generation are shadowed in gray.

III.2.4 Mutations not confirmed in the M3 generation

Seeds of selected M2 plants were sawn on soil and M3 plants were selected for the *msi1* mutation and analyzed for a reproducible silique size reduction. Sixteen lines (24, 73, 92, 93,105, 142, 269, 289, 446, 540, 550, 570, 647, 668, 938 and 1076) were excluded from the candidate mutant list since none of the member of the M3 generation exhibited the parental phenotype. The phenotype was indistinguishable from *msi1/MSI1; dde2/dde2* plants, suggesting that the growing conditions were probably not optimal during their growth in M2 generation.

For three lines 5, 207 and 952, the mutant M2 plants had strong plant morphology defects and flower homeotic mutation. Therefore, the study of those mutants was not carried further.

For three lines, 60, 440 and 693, mutant M2 plants with a reduction of silique size could not set seeds upon self fertilization. Seeds of family members, without silique size reduction i.e. likely to be heterozygous for the suppressor mutation, were sawn on soil. Plants were selected for the *msi1* mutation and the silique size reduction phenotype was indeed found again among the M3 plants. However, the mutant plants had strong plant morphology defects and flower homeotic mutation. Therefore, the study of those mutants was not carried further.

In the flowers of the mutants M2 plant of the line 187, no stamens were observed. The mutant had also a reduced number of ovules that accounted for the reduction of the silique length upon fis development

III.3 Classification of mutants

Few lines were confirmed in M3 generation to contain a secondary mutation affecting silique length elongation without fertilization. Table 3.2 shows the percentage of

plants impaired in silique elongation as well as the size of each candidate M3 family for the confirmed lines.

Family ID	M3	
	family size	% plants with reduced siliques
16	147	66.7
41	179	83.2
43	30	53.3

Table 3.2 Mutant lines with reproducible phenotype in the M3 generation.

III.3.1 Mutations affecting the female gametophyte

In mutant plants of family 43 flower morphology was normal. 25% of ovules underwent fis development (Fig 3.1 C). 25% were mature, normal ovules (Fig 3.1 B). However, 50% percent of ovules could not mature and suffered from an early developmental arrest (Fig 3.1 A), suggesting an early gametophytic defect. This hypothesis was further confirmed as I observed 50% of ovules growing after fertilization and a transmission of the mutant to 50% of the progeny (Table 3.2). As this mutation does not genetically interact with the *msi1* mutation and does not provide further insights into the interaction of seeds and fruits, no further studies were performed on this line.

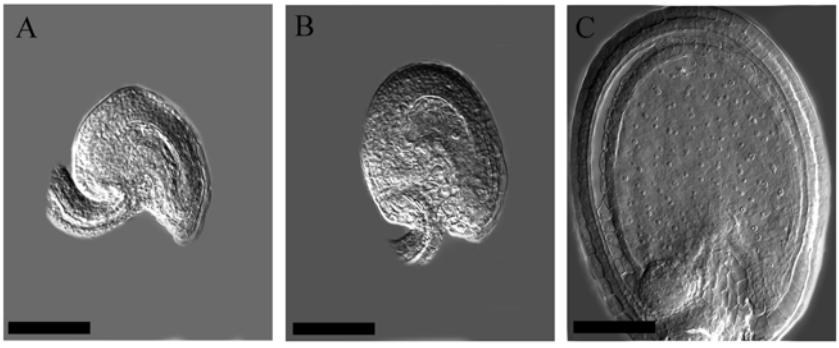

Fig 3.1 Mutation 43 causes an early defect of female gametophyte development. (**A**): immature ovule; (**B**): mature ovule; (**C**): ovule undergoing fis development. Scale bars: 50 μM.

III.3.2 Mutations affecting seed development

In families 16 and 41, 8.3 % (n=997) and 7.7 % (n=1114) of ovules underwent fis development, respectively. Two hypotheses could explain this phenotype: i) the suppressor mutations are physically linked to the *msi1* mutation or ii) the suppressor mutants are sporophytic mutants with a reduced penetrance. The first hypothesis predicts that the suppressor mutations should be heterozygous and all *msi1* ovules should be impaired in fis development. Therefore, only those ovules where the suppressor mutation segregated away from the *msi1* locus could still undergo fis development. According to this hypothesis, both loci would have a genetic distance of about 16 centiMorgans (cM) to the *msi1* locus, i.e. 16.64 +/- 1.24 cM for the suppressor mutation 16 and 15.44 cM +/- 0.19 cM for the suppressor mutation 41. The second hypothesis predicts that the suppressor mutations either need to be homozygous (if sporophytic recessive) or homo-or heterozygous (if dominant) on order to affect a majority of the *msi1* ovules. The penetrance of both suppressor mutations would be about 84 %.

III.4 Backcrosses of M2 plants

The M2 candidate plants were crossed as females with *msi1/MSI1;dde2/dde2* pollen donors. As the *msi1* allele is not maternally transmitted, *dde2/dde2* plants were used as females to backcross with M2 candidates in order to increase the size of progeny per cross. The progeny was selected for the *msi1* mutation.

	M2 x *msi1/MSI1,dde2/dde2*		*dde2/dde2* x M2	
FAMILY ID	family size	% plants with reduced siliques	family size	% plants with reduced siliques
16	5	0	100	81
41	8	0	26	84.6

Table 3.3 Transmission analysis of the fis suppressor phenotype. F1 plants were preselected for the *msi1* mutation.

Using mutants 16 and 41 as pollen donor to pollinate wild-type plants did not cause seed abortion, indicating that the suppressor mutations were not affecting embryogenesis in a heterozygous state when paternally introduced. When mutants 16 and 41 were pollinated with pollen from *msi1/MSI1;dde2/dde2* plants, 50% of seed abortion was observed (n=369). Thus, although both mutants can suppress autonomous seed development of *msi1* mutant gametophytes; they cannot alleviate the *msi1* seed abortion phenotype.

The progeny of wild-type plant pollinated with pollen from mutants 16 or 41 was composed of 311 and 70 plants before selection for the *msi1* allele, respectively. Almost one third of the progeny survived i.e. inherited of the *msi1* paternal mutant allele (Table 3.3). Among the *msi1*-selected progeny, more than 80% of the plants had reduced silique length (Table 3.3), indicating that mutations 16 and 41 were physically linked to the *msi1* locus.

The progeny of mutant 16 or 41 plants pollinated with *msi1/MSI1; dde2/dde2* pollen was composed respectively of 13 and 24 plants before selection for the *msi1* allele. Almost half of the progeny inherited of the *msi1* paternal mutant allele (Table 3.3). Among the *msi1*-selected progeny, none of the plants had reduced silique length (Table 3.3), confirming that mutations 16 and 41 were linked to the *msi1* locus.

The focus of this screen was to obtain a suppression of the fis phenotype i.e. the suppression of autonomous seed development. Both mutants 16 and 41 fit our criteria and are likely mutated at a single locus. Therefore, suppressor mutants 16 and 41 were studied more in detail. Both suppressor mutations possessed similarities. The majority of the F1 plants arising from the cross *dde2/dde2* x mutant16 or 41 that contained the *msi1* mutation also contained the *msi1* suppressor mutations, strongly suggesting that both suppressor mutations 16 and 41 were not only genetically but also physically linked to the *msi1* locus. The two suppressors were called *mod* for modifier of the *msi1* autonomous seed development. Both *mod* mutations, although not allelic, have overlapping phenotypes. Therefore, the data are shown only for *mod41*.

III.5 Mutants *mod* 16 and *mod* 41 are modifiers of the fis phenotype.

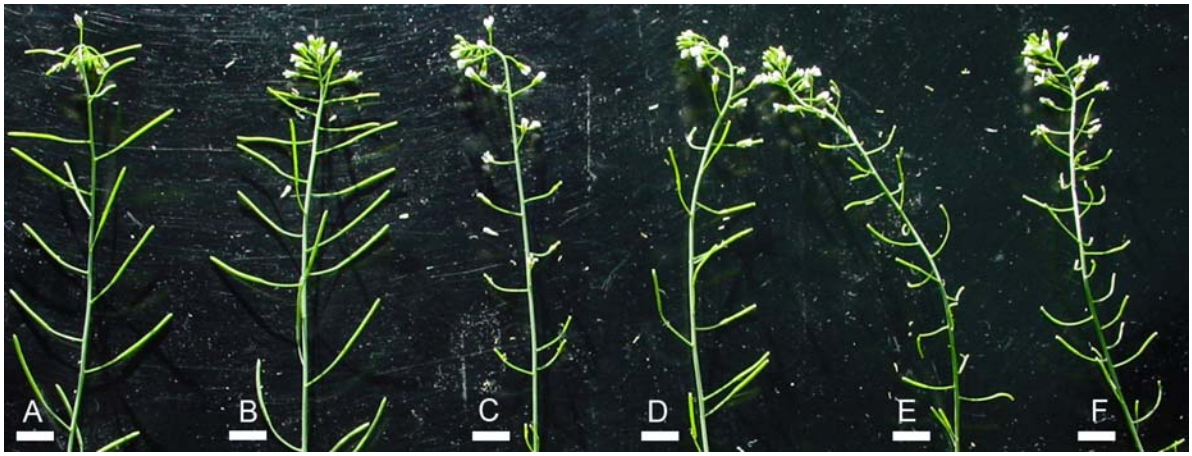

Fig 3.2 Mutations 16 and 41 suppress the *msi1* autonomous seed development phenotype. Picture of inflorescences of: (**A**) Columbia, (**B**) *msi1/MSI1*, (**C**) *dde2/dde2*, (**D**) *msi1/MSI1;dde2/dde2*, (**E**) *msi1/MSI1,mod16/MOD16;dde2/dde2,* (**F**) *msi1/MSI1,mod41/MOD41; dde2/dde2.* Scale bars: 1 cm.

III.5.1 Phenotypic analysis of *msi1, mod* mutants before fertilization

As shown in Figure 3.3 and Figure 3.4, both modifier loci *mod16* and *mod41* suppress the fis phenotype of *msi1* ovules. Without fertilization siliques of *msi1/MSI1* plants elongate up to 10 mm (Fig 3.2 and Fig 3.5), whereas unfertilized siliques of *msi1/MSI1,mod* plants elongated up to 6 mm (Fig 3.2 and Fig 3.5). Around 8 % of the ovules of both *mod* mutant lines underwent fis development in the absence of fertilization (Fig 3.3 and Fig 3.4 right). The remaining 92% remained as ovules-like structures. Ovules that underwent fis development are likely to be ovules in which recombination occurred, leading to the genotype *msi1,MOD.* Based on this recombination frequency, both *mod* mutations were estimated to be about 16 cM away from the *msi1* locus. Microscopic analysis of ovules of *msi1/MSI1,mod41/MOD41* plants revealed that 53% of the ovules were morphologically normal (Fig 3.4 left panel), however, 39 % of the ovules started fis development but arrested after a few mitotic divisions (n=254; Fig 3.4 middle panel). Therefore, the *mod* mutation suppressed the ability of *msi1* mutant ovules to undergo fis development, but did not prevent it completely.

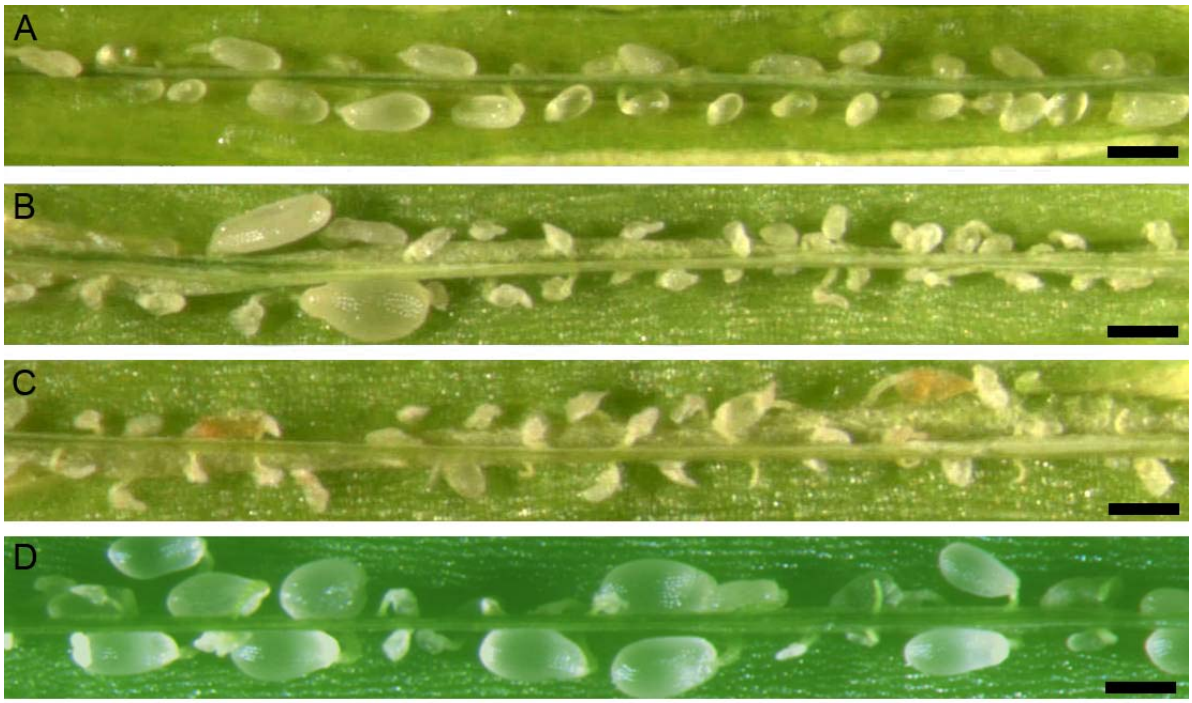

Fig 3.3 fis development in *msi1/MSI1, mod41/MOD41* plants. Open autonomously developing silique, different days after emasculation (dae). (**A**) silique 3-4 dae, (**B**) silique 9-10 dae, (**C**) silique 15-16 dae (**D**) control *msi1/MSI1* silique 9-10 dae. Scale bars: 100 μm.

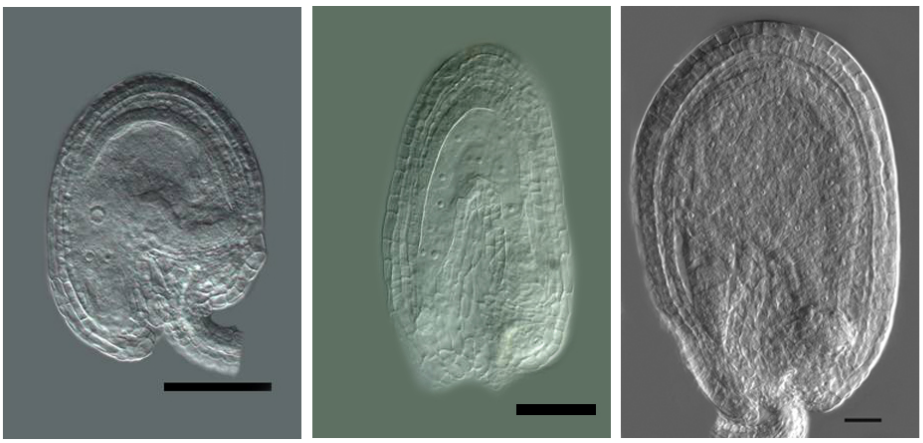

Fig 3.4 fis development is impaired in *msi1/MSI1, mod41/MOD41*. Left panel: wild-type ovule, middle panel: autonomously developing *msi1/mod41* ovules arrested after few mitotic cycles, right panel: autonomously developing *msi1* ovule. Scale bars 50 μm.

III.5.2 Phenotypic analysis of *msi1,mod* mutants after fertilization

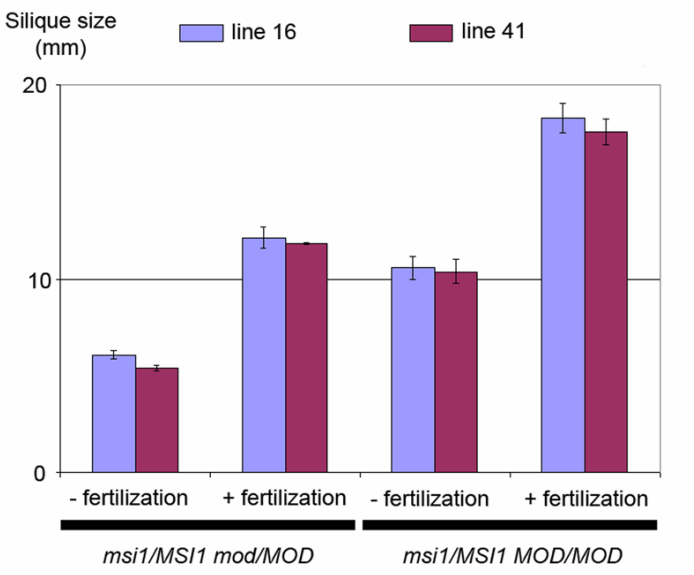

Fig 3.5 Silique length of *mod/MOD,msi1/MSI1* plant with or without fertilization. The length of at least 15 siliques were measured for each genotype. As *msi1*control, the plants without silique length reduction from the line 16 and 41 were used.

Upon self fertilization *msi1/MSI1* plants have 50% mature seeds, and 50% aborting seeds. Among the aborting seeds 17% were aborting early and 33% relatively late at the heart stage (Table 3.4). In an *msi1/MSI1, mod41/MOD41* self fertilized plant (Table 3.4), 38.4% of very early aborting seeds could be observed, indicating a genetic interaction between the two mutations (Fig 3.6 and Fig 3.7). The new class of early aborting seeds was correlated with a reduction of silique size to 12mm (Fig 3.2 and Fig 3.5) from 18 mm in an *msi1/MSI1* (Fig 3.2 and Fig 3.5) or in a wild-type plant (Fig 3.2).

When *msi1/MSI1, mod41/MOD41* plants were crossed with *msi1/MSI1* pollen, the distribution of the aborting seeds was similar to the ratio found in *msi1/MSI1* plants upon self fertilization (Table 3.5 and Fig. 3.6). This absence of ovule-sized seeds indicated that only *mod/mod* seeds being homo- or heterozygous for the *msi1* mutation strongly impaired seed development. In conflict with this assumption is the high frequency of ovule-sized seeds among aborting seeds (75%, table 3.4) in self fertilized *msi1/MSI1, mod41/MOD41*

plants. If we assume that the *mod* mutation rescues the paternal *msi1* transmission defect (Chapter 4, VI.2.1), we would expect maximally 50% ovule-sized seeds among the aborting seeds. One possible explanation for the significant higher number of ovule sized seeds is a preferential fertilization of *msi1/mod* mutant female gametophytes by *msi1/mod* mutant pollen. Clarification of this discrepancy needs further investigations.

Genotype	*msi1 /MSI, mod41/MOD41*	*msi1/MSI1*
total number	670	1009
% seeds	48.3	49.6
% seeds aborted at heart stage	13.3	32.8
% seeds aborted at globular stage	0	15.8
% ovule sized structures	38.4	0

Table 3.4 Seed abortion ratios upon self fertilization in *msi1/MSI1* and *msi1/MSI1, mod41/MOD41* plants. Seeds were counted 15 days after fertilization.

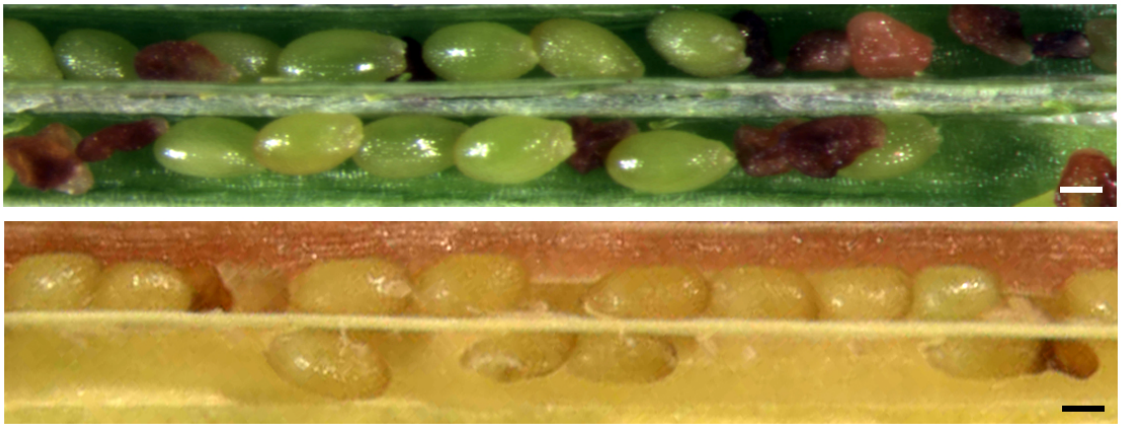

Fig 3.6 Seed development in *msi1/MSI1, mod41/MOD41*. Upper panel: open silique of a self-fertilized *msi1/MSI1* mutant plant at late embryogenesis. Lower panel: open silique of a self fertilized *msi1/MSI1, mod41/MOD41* mutant plant at late embryogenesis. Scale bars: 200 μm

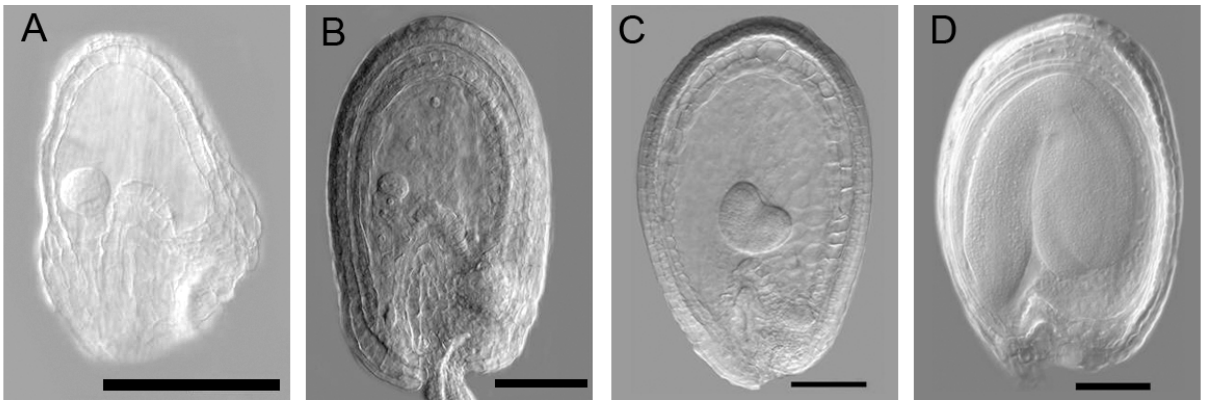

Fig 3.7 Seed development in the *msi1, mod41* background. (**A**) early aborted homozygous *msi1,mod41* seeds; (**B**) early aborted homozygous *msi1* seeds; (**C**) late aborted heterozyogous *msi1/MSI1* seeds; (**D**) maturing wild-type seed. Scale bar 100 μM.

Genotype	*msi1/MSI1, mod41/MOD41* x *msi1/MSI1*	*msi1/MSI1* x *msi1/MSI1, mod41/MOD41*
Total number	705	532
% seeds	52.1	52.2
% seeds aborted at heart stage	34	33.1
% seeds aborted at globular stage	13.9	14.7
% ovule sized structures	0	0

Table 3.5 Seed abortion ratios after reciprocal crosses of *msi1/MSI1* plants and *msi1/MSI1, mod41/ MOD41* plants. Seeds were counted 15 days after fertilization.

III.5.3 *mod16* and *mod41* are not allelic

Similar to the *mod41* mutation, I only observed ovule-sized seeds in self-fertilized *msi1/MSI1;mod16/MOD16* plants, whereas no ovule-sized seeds were observed in outcrosses of *msi1/MSI1, mod16/MOD16* with *msi1/MSI1* plants. Therefore, I used this

phenotype of ovule-sized seed formation to test whether both mutants are allelic. However, neither in crosses of *msi1/MSI1, mod41/MOD41* with pollen of *msi1/MSI1, mod16/MOD16* nor in the reciprocal cross, ovule-sized seeds could be observed, indicating that despite their similar properties *mod16* and *mod 41* are not allelic.

III.5.4 Phenotypic analysis of the *mod* phenotype in a wild-type background

In order to obtain *mod* mutant plants without the *msi1* mutation, I pollinated *msi1/MSI1, mod41/MOD41; dde2/dde2* plants with wild-type pollen. The progeny was allowed to self-fertilize and seed abortion was analyzed. Among 20 F1 plants three F1 plants were identified with one quarter of the seeds aborting. The seed abortion ratio is 27.35% (n= 892), suggesting that *mod41* is a zygotic recessive embryo lethal mutation (χ^2=0.12198). Furthermore, this indicates that the *mod41* mutation alone does not cause a preferential fertilization of *mod41* female gametophytes.

At a stage when wild-type seeds turned green, mutant *mod41/mod41* seeds remained white and finally collapsed (Fig. 3.8, lower panel). The average size of an aborting seed before collapsing was comparable to a wild-type seed of the same age, despite the fact that the mutant embryo did not reach the globular stage (Fig 3.8, upper right panel).

Similar to *mod41*, *mod16* was also embryo lethal and crossing of both *mod* mutants resulted in 100% mature seed formation, confirming that both mutations are not allelic. An alternative but unlikely explanation might be that both mutant 16 and 41 are causing an intragenic complementation.

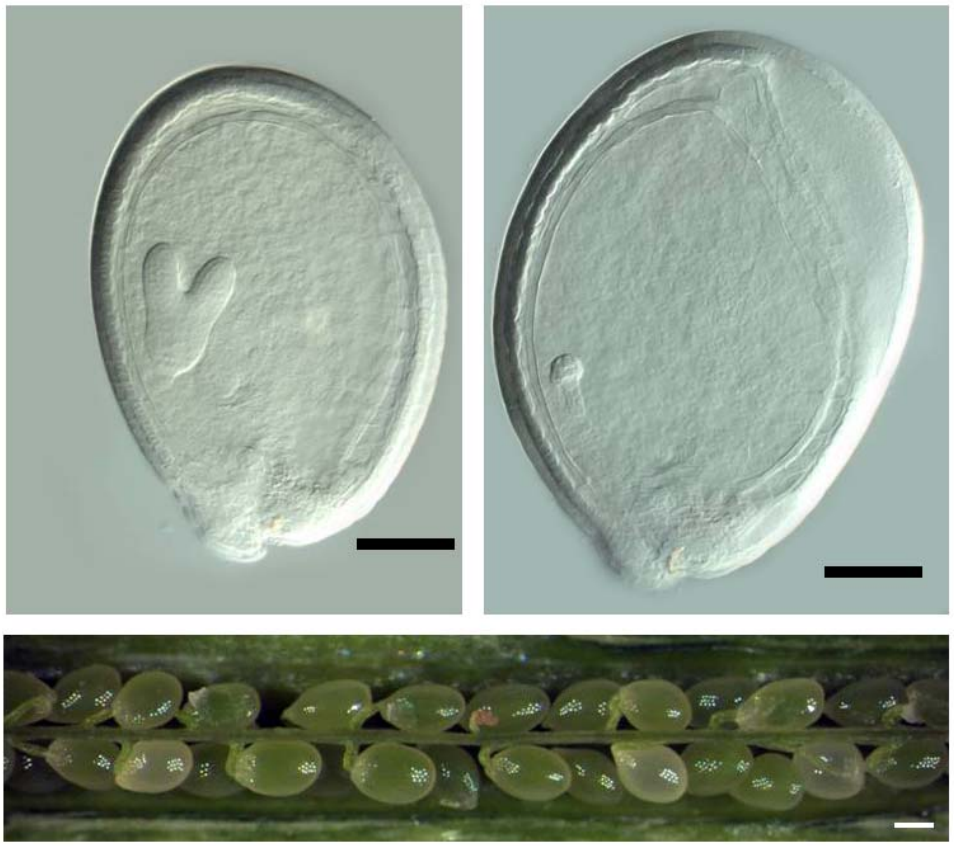

Fig 3.8 Seeds development in *mod41/MOD41* plants. Upper left panel: 75% of the seeds showed no developmental defect, right panel: 25% of the seeds aborted before reaching the globular stage, lower panel: Open silique twelve days after fertilization. Scale bars: in upper panels 100µM, in lower panels 200µM .

III.5.5 Phenotypic analysis of *fis2;mod41* double mutant

MSI1 is not only a subunit of the FIS complex but of several other complexes as well. Therefore, I wanted to determine whether the genetic interaction between *mod41* and *msi1* would also be observed in another *fis* mutant background. For this purpose, I introduced the *mod41* mutation into the *fis2* mutant background by crossing a *msi1/MSI1, mod41/MOD41* plant with pollen of a *fis2/FIS2* plant. Sixty F1 plants were recovered in which almost half of the plants were wild-type and the other half carried the *fis2* mutation. Among the *fis2*

mutant plants I observed three plants with a higher seed abortion ratio was 61.11 % (n=777 (χ^2=0.6768), Fig 3.9). This high ratio of aborting seed can be explained by independent segregation of the embryo lethal effect of the *mod41* mutation and the female gametophytic effect of the *fis2* mutation (Table 3.6).

+fert	*mod41*		*MOD41*	
	fis2	*FIS2*	*FIS2*	*fis2*
mod41	37.5% aborted		37.5% seeds	25% aborted
MOD41				

Table 3.6 Expected seed abortion ratio in a *mod41/MOD41; fis2/FIS2* plant is 62.5%.

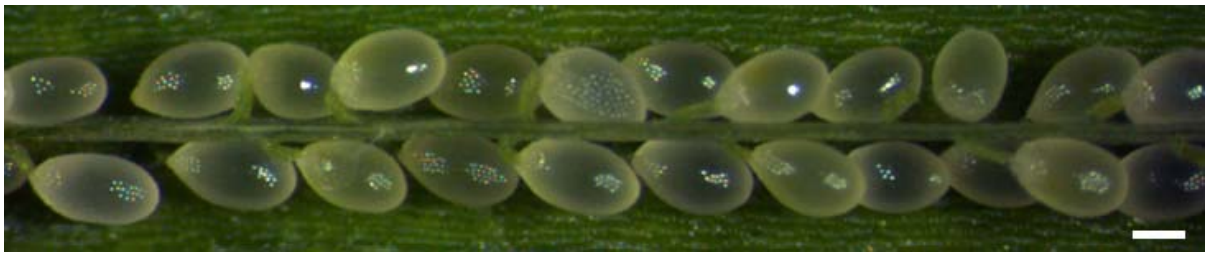

Fig 3.9 Open silique of a self-fertilized *mod41/MOD41; fis2/FIS2* plant. Scale bar 200 μm

CHAPTER IV POLYCOMB GROUP PROTEINS FUNCTION IN THE FEMALE GAMETOPHYTE TO DETERMINE SEED DEVELOPMENT IN PLANTS

IV.1 Introduction

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Mutations in *FIS* genes cause parent-of-origin-dependent seed abortion. All seeds that inherit a mutant *fis* allele from the mother abort, regardless of the presence of a wild-type paternal allele. Development of *fis* mutant seeds is delayed, and seeds abort with embryos arrested at the late heart stage and non-cellularized endosperm with strongly overproliferated chalazal endosperm domains (Grossniklaus et al., 1998; Kiyosue et al., 1999; Köhler et al., 2003b; Guitton et al., 2004). The maternal effect parent-of-origin-dependent seed abortion in *mea* and *fis2* mutants could be explained by the findings that *MEA* and likely *FIS2* are imprinted genes, with the paternal allele of both genes being specifically silenced in the seed (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000; Jullien et al., 2006). Similarly, the paternal *FIE* allele is not expressed during early stages of seed development, providing an explanation for the maternal effect of *fie* mutants (Yadegari et al., 2000).

It is likely that MEA and FIS2 are subunits specific for the FIS complex, whereas FIE and MSI1 are part of several distinct PRC2-like complexes (Schubert et al., 2005; Hennig, 2005). Furthermore, MSI1 is potentially part of several different complexes, such as Chromatin Assembly Factor CAF-1, histone deacetylases and chromatin remodeling machines that are likely to play a role during early embryogenesis (Hennig, 2005). Similar to *mea*, *fis2* and *fie* mutants, lack of MSI1 function causes parent-of-origin dependent seed abortion. However, it has been proposed that the lack of MSI1 function causes, in addition to the gametophytic effect, also a sporophytic effect on seed development (Guitton et al., 2004). Thus, lack of both, maternal and paternal *MSI1* alleles causes a significantly stronger defect than the lack of the maternal *MSI1* allele alone. This implies that the paternal allele of *MSI1* is active, but fails to complement the maternal gametophytic *msi1* defect.

To test this idea, I investigated the temporal requirements of MSI1 during seed development. I specifically addressed the question, whether early paternal expression of *MSI1* is sufficient to rescue the maternal effect *msi1* seed abortion phenotype.

IV.2 RESULTS

IV.2.1 Loss of *MSI1* causes gametophytic and sporophytic effects on seed development

Complete loss of *MSI1* is lethal, and the *msi1*allele used in this study can only be maintained in heterozygous *msi1/MSI1* plants (here referred to as *msi1* plants) (Köhler et al., 2003b; Guitton et al., 2004). Self-fertilized *msi1* mutant plants form two classes of aborting seeds: an early aborting class, which contains grossly abnormal embryos, and a late aborting class, which contains embryos that phenotypically closely resemble *fis*-class mutant embryos (Köhler et al., 2003b; Guitton et al., 2004). It has been suggested that lack of MSI1 function has a gametophytic as well as a sporophytic zygotic effect, causing the formation of early and late aborting seeds, respectively (Guitton et al., 2004). This model predicts that 50% of the seeds inherit a maternal *MSI1* allele and develop normally; 50% of the seeds inherit a maternal *msi1* allele and abort early if also inheriting a paternal *msi1* allele or abort late if inheriting a paternal *MSI1* allele.

I observed that the *msi1* allele has 17% early aborting (Fig. 4.2 A and C) and 33% late aborting seeds (Fig. 4.1 A and C), which deviates from the ratio of 25% early to 25% late aborting seeds predicted by the model (n=583, χ^2=29.72 >$\chi^2_{0.05[2]}$=5.991; Fig. 4.1 C). One reason for this discrepancy could be a reduced transmission of the paternal *msi1* allele. I tested this hypothesis by determining the transmission of the *msi1* allele through pollen. Indeed, I found that the transmission of the paternal *msi1* allele is reduced to 72% (n=500). Taking the reduced transmission of the paternal *msi1* allele into account, only 18%

homozygous *msi1* mutant seeds can be expected. This number closely matches the observed number of 17% early aborting seeds.

To unequivocally test the hypothesis that early aborting seeds require a paternally inherited *msi1* allele, I pollinated heterozygous *msi1* mutant plants with wild-type pollen. In this experiment, 51% of the seeds were phenotypically wild-type and 49 % of the seeds were late aborting with a fis-like phenotype (1 wild type : 1 *msi1/MSI*, n=487, $\chi^2=0.166<\chi^2_{0.05[1]}=3.841$; Fig. 4.1 B and C). I did not observe any early aborting seeds, clearly demonstrating that loss of both, maternal and paternal *MSI1* alleles, is the prerequisite for early seed abortion. This result suggests that the paternal *MSI1* allele is expressed and, consequently, that *MSI1* is not regulated by genomic imprinting, in contrast to the *fis*-class gnes *MEA* and *FIS2*.

IV.2.2 *MSI1* is paternally expressed in embryo and endosperm

To test the hypothesis that *MSI1* has bi-allelic expression, I examined expression of maternally and paternally inherited *MSI1* alleles during early seed development. I made use of a mutant containing a silent mutation in the *MSI1* coding region (referred to as *MSI1**). I developed a specific PCR assay that allowed to distinguish the *MSI1** allele from the wild-type *MSI1* allele. As shown in Figure 4.2 A, primers s1 and as1, which were designed for the wild-type *MSI1* allele, did not amplify the *MSI1** allele. Conversely primers s2 and as1, which were designed for the *MSI1** allele, did not amplify the *MSI1* allele. I performed reciprocal crosses between wild-type and *MSI1** plants and tested the expression of the paternally inherited *MSI1* allele. Regardless whether wild-type plants or *MSI1** plants were used as pollen donors, I could clearly detect expression of the paternal *MSI1* allele starting three days after pollination (DAP, Fig. 4.2 B). Thus, timing of paternal *MSI1* expression is comparable to the majority of paternal alleles (Vielle-Calzada et al., 2000). As *MSI1* is also expressed in sporophytic tissues (Hennig et al., 2003; Köhler et al., 2003b) transcripts of maternal *MSI1* or *MSI1** alleles are contributed by zygotic tissues as well as maternal

sporophytic tissues. Therefore, the maternal alleles yielded always signals of higher intensity than the paternal alleles.

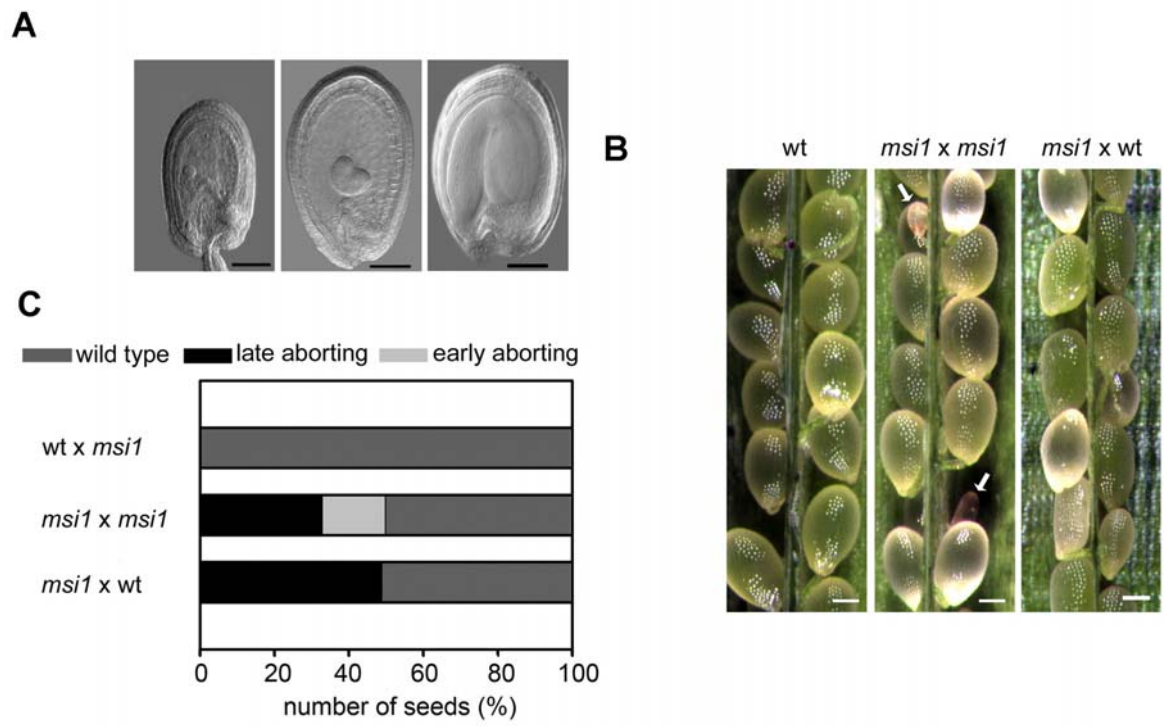

Fig. 4.1 Homozygous *msi1* mutant seeds show an early developmental arrest.
(**A**) Cleared seeds derived from the same silique that arrested at different developmental stages. Homozygous *msi1* seeds (left panel), heterozygous *msi1* seeds (middle panel) and wild-type seeds (right panel). (**B**) Self-pollinated *msi1/MSI1* plants form small aborting seeds (middle panel). No small aborting seeds are formed after pollination of *msi1/MSI1* plants with wild-type pollen (right panel). A wild-type (wt) silique is shown as a control in the left panel. Arrows indicate small aborting seeds. (**C**) Quantification of seed abortion observed after crosses of wild-type x *msi1*/*MSI1* (n=212), *msi/MSI1* x *msi1/MSI1* (n=583) and *msi1/MSI1* x wt (n=487). Scale bars: 100 µM in A, 200 µM in B.

Imprinting of several genes has been shown to occur specifically in the endosperm, and the same genes were biallelically expressed in the embryo (Haun et al., 2007; Kinoshita et al., 1999; Kinoshita et al., 2004). *MSI1* is expressed in the embryo and endosperm (Köhler et al., 2003b). Therefore, I investigated whether expression of the paternal *MSI1* allele is confined to the embryo and *MSI1* is imprinted in the endosperm, or whether the

paternal *MSI1* allele is also expressed in the endosperm. For this purpose I performed crosses of wild-type plants with *MSI1** plants and dissected F1 seeds at 6 DAP into embryo and endosperm plus seed coat fractions. As shown in Figure 4.2 C, I could clearly detect expression of the paternal *MSI1** allele in the embryo as well as in the endosperm.

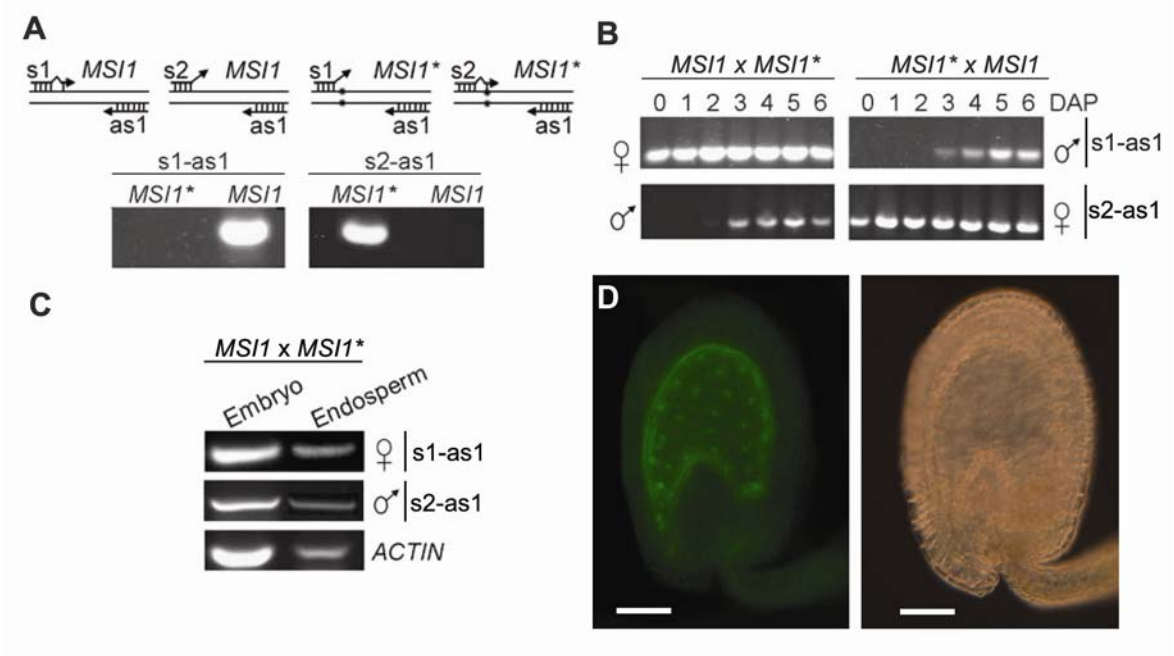

Fig. 4.2 The paternal *MSI1* allele is expressed in the embryo and endosperm. (**A**) Schematic presentation of the PCR assay used to amplify specifically either the *MSI1* or *MSI1** allele (upper panel). Primer combinations s1-as1 amplify only the *MSI1* allele (lower, left panel), primer combination s2-as1 amplifies only the *MSI1** allele (lower, right panel). (**B**) Time course analysis of maternal and paternal *MSI1* expression. Reciprocal crosses of wild-type (*MSI1*) and *MSI1** plants were performed, and expression of maternal and paternal alleles was analyzed by RT-PCR in siliques derived from these crosses. The upper panel shows results from primer combination s1-as1, the lower panel shows results from primer combination s2-as1. (**C**) Seeds derived from a cross of *MSI* and *MSI1** plants were dissected at 6 DAP. Embryos and endosperm plus seed coat fractions were analyzed for expression of maternal (*MSI1*) and paternal *MSI1** alleles by RT-PCR (**D**) Left panel: Fluorescent micrograph of wild-type seed pollinated with *MSI1::GFP* pollen at 2 DAP. Right panel: Corresponding light micrograph. Scale bars: 50 μm.

To obtain independent evidence that *MSI1* was paternally expressed in the endosperm, a vector was constructed, in which the *MSI1* promoter was fused to the *MSI1* cDNA that was fused in frame with the *GREEN FLUORESCENT PROTEIN* (*GFP)* sequence (referred to as *MSI1::GFP*). Among five primary transformant lines in the *msi1* mutant background, two lines completely rescued the *msi1* seed abortion phenotype and showed fluorescence in the female gametophyte. I used one of the complementing *MSI1::GFP* line to pollinate a wild-type plant. Expression of the paternal allele in the endosperm was detected already two days after pollination (Fig. 4.2 D). This is one day earlier compared to the PCR based assay, which might be caused by a different expression of the *MSI1::GFP* transgene compared to the endogenous gene or by the low amounts of paternal transcript at 2 DAP that escaped the PCR-based detection. However, both experiments support the conclusion that *MSI1* is not imprinted, but biallelically expressed in both, embryo and endosperm.

IV.2.3 The female gametophytic defect of *msi1* mutants does not affect embryo patterning

Heterozygous *msi1* mutant seeds abort with embryos arrested at the late heart stage and strongly overproliferated chalazal endosperm domains (Köhler et al., 2003b; Guitton et al., 2004). However, it remains elusive why *msi1* mutant embryos arrest development and abort despite expression of the paternal *MSI1* allele. It is possible that developmental defects start to accumulate early during embryogenesis when most of the paternal genome including *MSI1* is still inactive and cause severe developmental abnormalities later in embryogenesis culminating in seed abortion. Therefore, I tested whether marker genes that define major developmental steps during early embryogenesis are correctly expressed in *msi1* mutant embryos compared to wild-type embryos. I tested markers for auxin distribution *DR5*; (Friml et al, 2003), the developing suspensor *WUSCHEL-related homeobox 8* (*WOX8*; Haecker, et al., 2004), provascular tissue (enhancer trap line Q0990; Weijers et al., 2006), the quiescent center *SCARECROW*; (*SCR*; Wysocka-Diller et al., 2000), and cells within the region to form the shoot apical meristem (enhancer trap lines

M0221 and M0223; Cary et al., 2002). The enhancer trap lines are publicly available from the Haselhoff collection (http://www.plantsci.cam.ac.uk/Haseloff/construction/catalogFrame.html).

The marker for auxin distribution *DR5::GFP* (Friml et al., 2003), was tested at the heart stage for wild-type and *msi1* embryos (Fig. 4.3). In both embryos, the auxin-reporting *DR5::GFP* marker was confined to the root pole, cotyledon tips and provascular tissue, indicating that the auxin distribution was not affected in *msi1* embryos.

For the cell identity of the basal area of the embryo i.e. the suspensor, the marker *WOX8::YFP* was investigated (Fig. 4.3). Expression of the reporter *WOX8::YFP* was confined to the suspensor in wild-type and in *msi1* mutant embryos (Haecker et al., 2004), indicating that the basal derivatives of the zygote forming the suspensor are correctly established.

The provascular tissue marker from enhancer trap line Q0990 is expressed in provascular cells of the central region immediately adjacent to the hypophysis (Weijers et al., 2006) Because this expression pattern remained in *msi1* (Fig. 4.3), specification of provascular cells seems to occur properly in *msi1* mutant embryos.

Establishment of root apical meristems was monitored using *SCR::YFP*, which is expressed only in the quiescent center and derivatives of the ground meristem (Wysocka-Diller et al., 2000). Expression of *SCR::YFP* in *msi1* closely resembled expression in wild-type (Fig. 4.3), suggesting that initiation of the root apical meristem is mostly normal in *msi1*.

To monitor formation of shoot apical meristems, enhancer trap lines M0221 and M0223 were used (Fig. 4.3). Both lines show GFP reporter activity in cells within the region forming the shoot apical meristem; and M0223 reflects expression of *CUP SHAPED COTYLEDON 1* (*CUC1*) (Cary et al., 2002). Similar to the other markers used, activity of M0221 and M0223 was similar in the wild type and *msi1*, indicating that progenitor cells for the shoot apical meristem are properly specified.

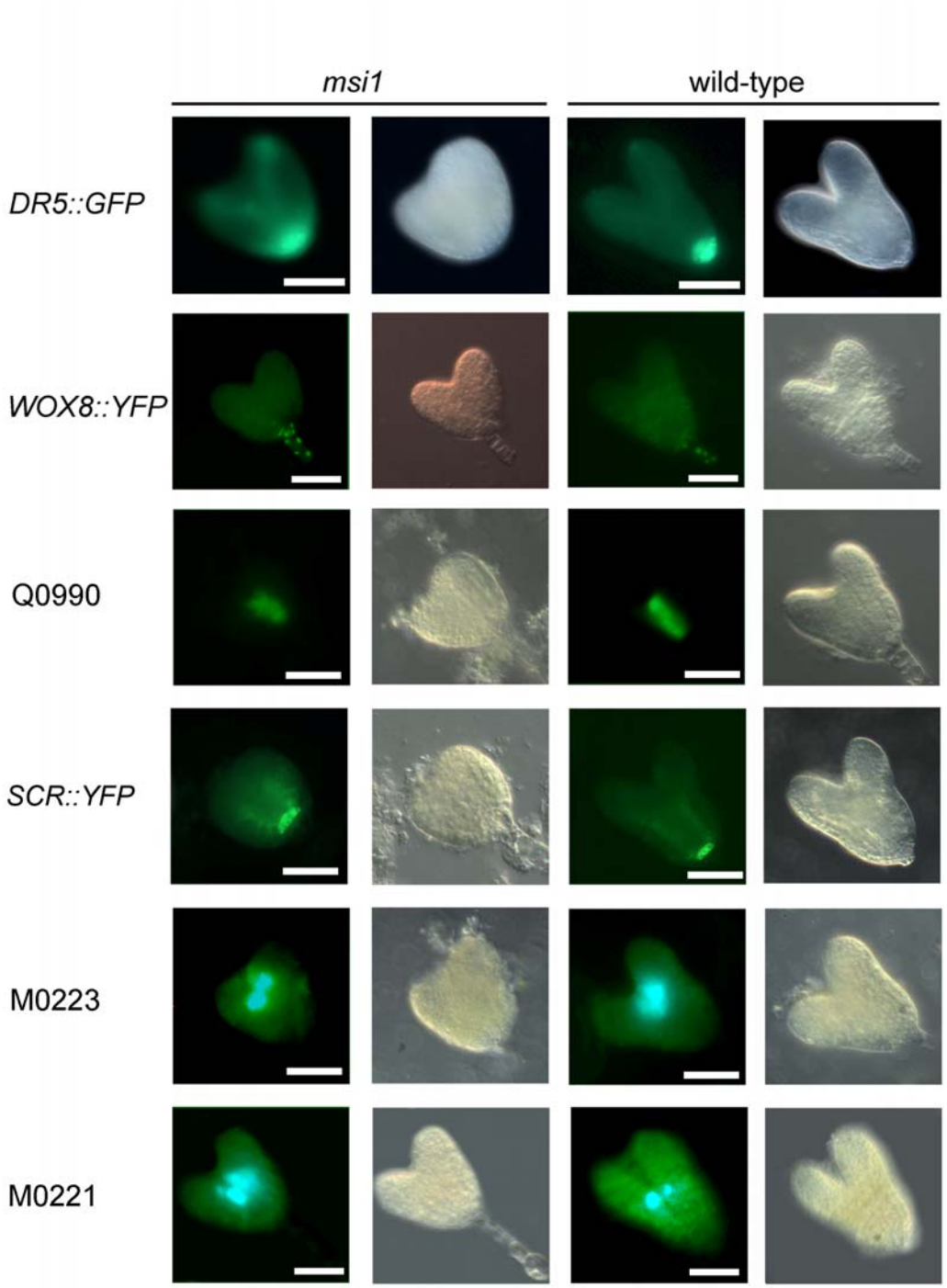

Fig. 4.3 Markers for embryo pattern formation are similarly expressed in wild-type and *msi1* mutant embryos. Expression of *DR5::GFP*, *WOX8::YFP*, Q0990, *SCR::YFP*, M0223 and M0221 in *msi1* mutant and wild-type embryos. Corresponding bright-field images of embryos are shown on the right panels. Scale bars: 50 μM.

Based on these findings I conclude that the defects established in the *msi1* female gametophyte or in early embryogenesis did not affect basic embryo pattern formation and embryo arrest at the late heart stage was caused by mechanisms that remain to be identified.

IV.2.4 *MSI1* is essential before fertilization

I considered three possible explanations for the female gametophytic defect of *msi1* mutants: (i) insufficient expression of the paternal *MSI1* allele at 3 DAP (Fig. 4.2 B) may be responsible for the maternal gametophytic effect, (ii) delayed expression of the paternal *MSI1* allele at 3 DAP (Fig. 4.2 B) may be responsible for the maternal gametophytic effect, or (iii) lack of functional MSI1 causes a defect in the female gametophyte and the consequences of this defect become obvious during later stages of seed development.

Paternal overexpression of *MSI1* after fertilization cannot rescue the *msi1* female gametophytic defect

I tested the first possibility by overexpressing paternal *MSI1* using the Cauliflower mosaic virus *35S* promoter (*35S*). I established transgenic plants containing the *35S* promoter fused to the *β-GLUCORONIDASE* (*GUS*) reporter gene (referred to as *35S::GUS*) and investigated the paternal expression pattern of this reporter gene construct by crossing wild-type plants with pollen from *35S::MSI1* transgenic plants. GUS expression started in the whole seed about 2 DAP (Fig 4.4 A).

Then, I established transgenic lines containing the *35S* promoter fused to the *MSI1* coding sequence (referred to as *35S::MSI1*). Using these lines, I addressed the question whether paternal overexpression of *MSI1* could rescue the seed abortion phenotype. In heterozygous *msi1* plants, 50% of the seeds abort as they carry a maternal *msi1* allele. If paternal overexpression of *MSI1* could rescue this female gametophytic *msi1* phenotype, I expected that a hemizygous *35S::MSI1* construct could rescue 25% of the seeds leading to 75% normal seeds. However, among 15 independent transgenic *35S::MSI1* lines in an *msi1*

mutant background, I did not identify any plant with more than 50% normal seeds indicating that *MSI1* paternal overexpression is not sufficient to rescue the gametophytic *msi1* mutant defect. Instead, I observed a reduction of the number of early aborting seeds by about half, suggesting that paternally overexpressed *35S::MSI1* is sufficient to promote development of early aborting homozygous *msi1* mutant seeds up to the stage of late aborting heterozygous *msi1* seeds (data not shown).

This hypothesis was tested by pollinating *msi1* mutant plants with pollen of two independent homozygous *35S::MSI1* transgenic lines in an *msi1* mutant background and scoring subsequent seed development. In contrast to pollination with pollen from heterozygous *msi1* plants, resulting in 17% early aborting seeds, after pollination with pollen from homozygous *35S::MSI1* transgenic plants, no early aborting seeds were observed (Fig. 4.4 B and D). Thus, paternal *MSI1* overexpression is sufficient to prolong development of homozygous *msi1* mutant seeds. I analyzed seeds of this cross by clearing and found no significant change of seed development compared to seeds developing on *msi1* plants pollinated with wild-type pollen (Fig. 4.4 C).

To unequivocally test whether paternal *MSI1* overexpression can rescue the *msi1* mutant phenotype I tested transmission of the maternal *msi1* allele after pollination of *msi1* plants with pollen of *35S::MSI1* plants. I crossed *msi1* plants with pollen of two independent *35S::MSI1* transgenic lines in a wild-type background. I scored the transmission of the maternal *msi1* allele to the progeny with a resistance gene associated to the *msi1* mutation. Testing more than 250 seedlings for each line, I found no significant maternal transmission of *msi1* (Table 4.1). Thus, I conclude that paternal *MSI1* overexpression is not sufficient to rescue the female gametophytic defect of *msi1* mutant seeds.

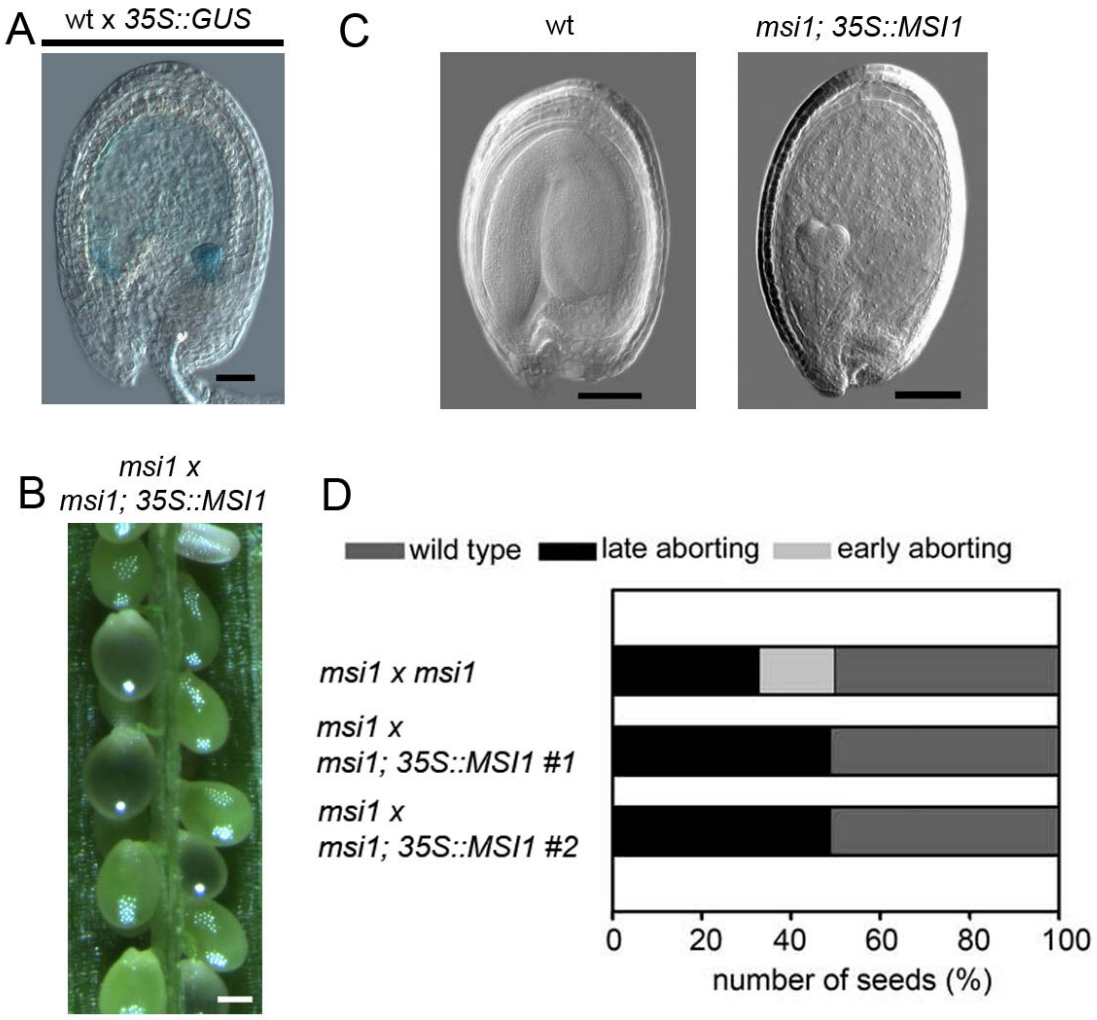

Fig. 4.4 Overexpression of *MSI1* after fertilization does no rescue the *msi1* mutant phenotype. (**A**) Wild-type plant pollinated with *35S::GUS* pollen shows GUS expression in seeds at 2 DAP. (**B**) *msi1* mutants pollinated with *msi1/MSI1;35S::MSI1* pollen have 50% late aborting seeds. (**C**) Cleared seeds derived from a silique of an *msi1* mutant pollinated with *msi1;35S::MSI1* pollen. Left panel: Wild-type (wt) seed, Right panel: *msi1* mutant seed. (**D**) Quantification of seed abortion observed after crosses of *msi/MSI1* x *msi1/MSI1* (n=583) and *msi1/MSI1* x *msi1/MSI1;35S::MSI1* #1 (n=345), *msi1/MSI1* x *msi1/MSI1;35S::MSI1* #2 (n=326). Scale bars: 200 µM in B, 100 µM in C and 50 µM in A.

Paternal expression of *MSI1* immediately after fertilization cannot rescue the *msi1* female gametophytic defect

I tested the second possibility by expressing paternal *MSI1* immediately after fertilization. I made use of the *PHERES1* (*PHE1*) promoter that is one of the few promoters escaping early paternal silencing and is expressed immediately after fertilization (Köhler et al., 2005).

I tested whether expression of *MSI1* under control of the *PHERES1* promoter (referred to as *PHE1::MSI1*) could be detected immediately after fertilization by crossing wild-type plants with pollen derived from *PHE1::MSI1* transgenic plants. Indeed, expression of the paternal *MSI1* allele was detected at one day after pollination (DAP) at a similar expression level compared to the endogenous *MSI1* allele starting at 3 DAP (Fig 4.5 A). If early paternal expression of *MSI1* would rescue this female gametophytic *msi1* phenotype, I expected that a hemizygous *PHE1::MSI1* construct could rescue 25% of the seeds and lead to 75% normal seeds. However, among 11 independent transgenic *PHE1::MSI1* lines in an *msi1* mutant background, I did not identify any plant with more than 50% normal seeds, indicating that early paternal expression is not sufficient to rescue the gametophytic *msi1* mutant defect. Instead, I observed a reduction of the number of early aborting seeds by about half, suggesting that paternally expressed *PHE1::MSI1,* like the paternally expressed *35S::MSI1,* is sufficient to promote development of early aborting homozygous *msi1* mutant seeds up to the stage of late aborting heterozygous *msi1* seeds (data not shown).

This hypothesis was tested by pollinating *msi1* mutant plants with pollen of three independent homozygous *PHE1::MSI1* transgenic lines in an *msi1* mutant background and scoring subsequent seed development. In contrast to pollination with pollen from heterozygous *msi1* plants, which led to 17% early aborting seeds, pollination with pollen from *PHE1::MSI1* transgenic lines led to no early aborting seeds (Fig. 4.5 B, D). Thus, early paternal *MSI1* expression is sufficient to establish prolonged development of homozygous *msi1* mutant seeds. I analyzed seeds of this cross by clearing and found no

significant change of seed development compared to seeds developing on *msi1* plants pollinated with wild-type pollen (Fig. 4.5 C).

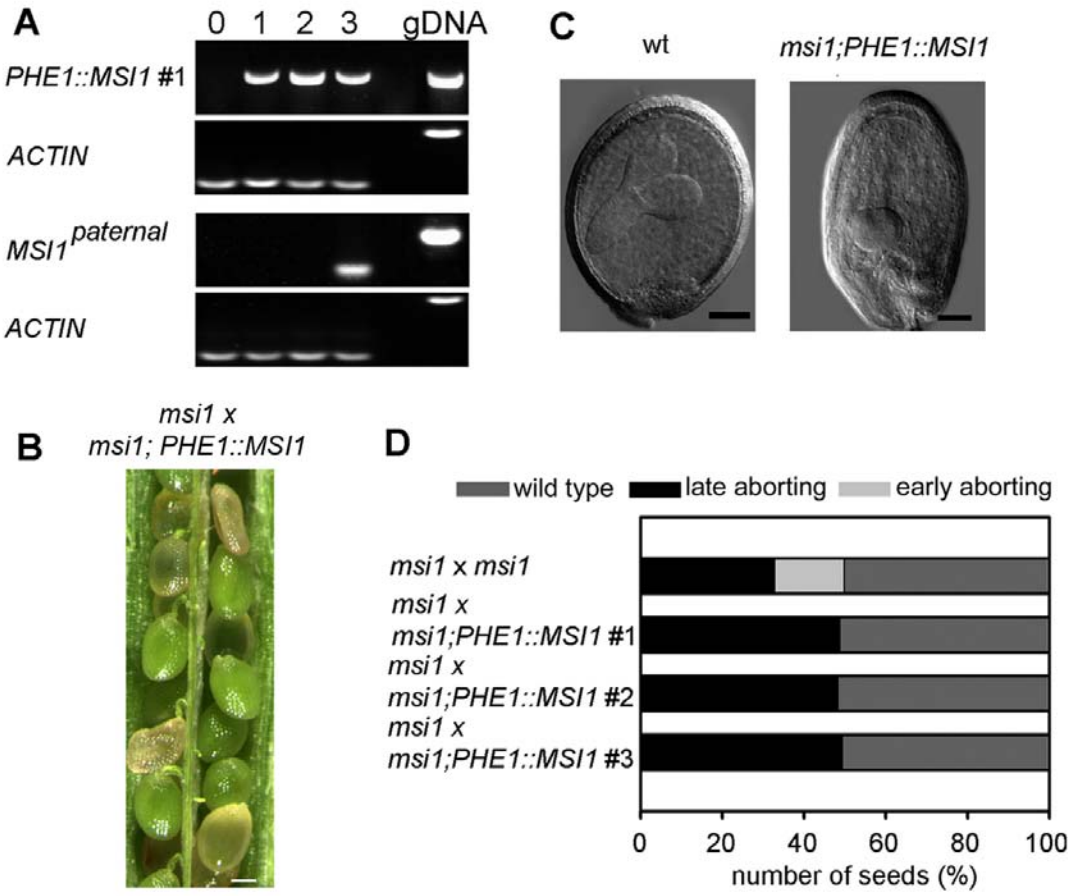

Fig. 4.5 Early paternal *MSI1* expression does no rescue the *msi1* mutant phenotype. (**A**) Early paternal expression of the *PHE1::MSI1* transgene was tested by RT-PCR in seeds derived from wild-type plants pollinated with *PHE1::MSI1* pollen. The primers detect specifically only the transgene-derived *MSI1* transcript. Paternal *MSI1* expression in seeds derived from *MSI1** plants pollinated with wild-type pollen is shown as a control. (**B**) *msi1* mutants pollinated with *msi1/MSI1;PHE1::MSI1* pollen have 50% aborted seeds. (**C**) Cleared seeds derived from the same silique of an *msi1* mutant pollinated with *msi1/MSI1;PHE1::MSI1* pollen. Wild-type (wt) seed (left panel), *msi1* mutant seed (right panel). (**D**) Quantification of seed abortion observed after crosses of *msi/MSI1* x *msi1/MSI1* (n=583) and *msi1/MSI1* x *msi1/MSI1;PHE1::MSI1* #1 (n=591), *msi1/MSI1* x *msi1/MSI1;PHE1::MSI1* #2 (n=882), *msi1/MSI1* x *msi1/MSI1;PHE1::MSI;* #3 (n=552). Scale bars: 200 µM in B, 50 µM in C.

To unequivocally test whether early paternal *MSI1* expression can rescue the *msi1* mutant phenotype I tested transmission of the maternal *msi1* allele after pollination of *msi1* plants with pollen of *PHE1::MSI1* plants. I crossed *msi1* plants with pollen of three independent *PHE1::MSI* transgenic lines in a wild-type background. Using more than 100 seedlings for each line, I found no significant maternal transmission of *msi1* (Table 4.1). Thus, I conclude that early paternal *MSI1* expression is not sufficient to rescue the female gametophytic defect of *msi1* mutant seeds.

Expression of *MSI1* in the female gametophyte can rescue the *msi1* female gametophytic defect

Because early paternal *MSI1* expression could not rescue the female gametophytic defect of *msi1* mutants, I addressed the question whether expression of *MSI1* in the female gametophyte could rescue the female gametophytic defect and restore wild-type seed development of *msi1*. The *DD46* promoter (At1g22015) has been shown to be active in the central cell and the synergid cells of the female gametophyte (Portereiko et al., 2006). I established transgenic plants containing the *DD46* promoter fused to the *β-GLUCORONIDASE* (*GUS*) reporter gene (referred to as *DD46::GUS*) and investigated the temporal and spatial expression pattern of this reporter construct. Before fertilization, I detected GUS activity in the central cell, in the synergids as well as in the egg cell. After fertilization, GUS expression ceased and was almost undetectable within the seed when the embryo had reached the globular stage, about 2 DAP (Fig 4.6 A). I confirmed this expression pattern using microarray data obtained from different reproductive stages of *Arabidopsis* development (Hennig et al., 2004). Whereas *DD46* is highly expressed before fertilization, no significant transcript levels are detectable after pollination (Fig 4.6 B). Thus, the *DD46* promoter is specifically active in the female gametophyte and expression ceases after fertilization.

I established transgenic lines containing the *DD46* promoter fused to the *MSI1* coding sequence (referred to as *DD46::MSI1*). Using these lines, I addressed the question whether expression of *MSI1* in female gametophytes of *msi1* mutants could rescue the seed abortion phenotype. I obtained 11 transgenic lines in an *msi1* mutant background and identified four lines with less than 50% seed abortion. Homozygous *DD46::MSI1* plants from two such transgenic lines in the heterozygous *msi1* background were pollinated with wild-type pollen and the F1 developing seeds were analyzed. I performed at least five crosses with each line, and in all instances I found a complete rescue of seed development (Fig 4.6 C and D). Microscopic analysis revealed that seed development was completed without any obvious phenotypical differences to wild-type seeds (Fig 4.6 E).

To obtain final proof that expression of *MSI1* in the female gametophyte can completely restore seed development of heterozygous *msi1* mutant seeds, I analyzed the transmission of the *msi1* mutant allele through the female gametes. I pollinated two independent *msi1/MSI1* transgenic lines hemizygous for *DD46::MSI1* with wild-type pollen and tested the F1 progeny resulting from this cross for the presence of the *msi1* mutant allele. Whereas the maternal *msi1* allele was never transmitted in non-complemented mutants seeds, most *msi1* gametophytes containing the *DD46::MSI1* construct could transmit the maternal *msi1* mutant allele (Table 4.1).

Finally, I tested whether *DD46::MSI1* could suppress autonomous endosperm development in *msi1* mutants. Therefore, I emasculated 13 flowers of two independent transgenic lines showing rescue of the *msi1* seed abortion phenotype and scored the development of the gametophytes six days after emasculation. Whereas the central cell of control *msi1* plants reproducibly underwent autonomous endosperm formation, all of the *msi1; DD46::MSI1* gametophytes arrested development after fusion of the polar nuclei (Fig 4.6 F, Table 4.2). Thus, I conclude that *DD46::MSI1* can completely rescue both aspects of the *msi1* mutant phenotype, seed abortion as well as autonomous endosperm development. According to the last hypothesis, *MSI1* is required in the female gametophyte to successfully rescue the aborting seed.

Line	Resistant	Non-resistant	Expected
msi1 x wild type	0	465 (100%)	100%
msi1/MSI1 x *35S::MSI1* #1	0	265 (100%)	0%
msi1/MSI1 x *35S::MSI1* #2	1	422 (99.7%)	0%
msi1/MSI1 x *PHE1::MSI1* #1	0	138 (100%)	0%
msi1/MSI1 x *PHE1::MSI1* #2	1	221 (99.5%)	0%
msi1/MSI1 x *PHE1::MSI1* #3	1	104 (99.0%)	0%
msi1/MSI1; DD46::MSI1/+ #1 x wild type	54	133 (71.1%)	66.7%
msi1/MSI1; DD46::MSI1/+ #2 x wild type	34	123 (78.3%)	66.7%

Table 4.1 Transmission analysis of the *msi1* mutant allele through the female gametophyte in different transgenic backgrounds.

Genotype	Seed-like	Ovules	n	Penetrance
wild type	0%	100%	324	0%
msi1/MSI1	49%	51%	224	98%
msi1/MSI1;DD46::MSI1 #1	0%	100%	756	0%
msi1/MSI1;DD46::MSI1 #2	0%	100%	663	0%

Table 4.2. Autonomous endosperm development in *msi1/MSI1; DD46::MSI1* transgenic lines.

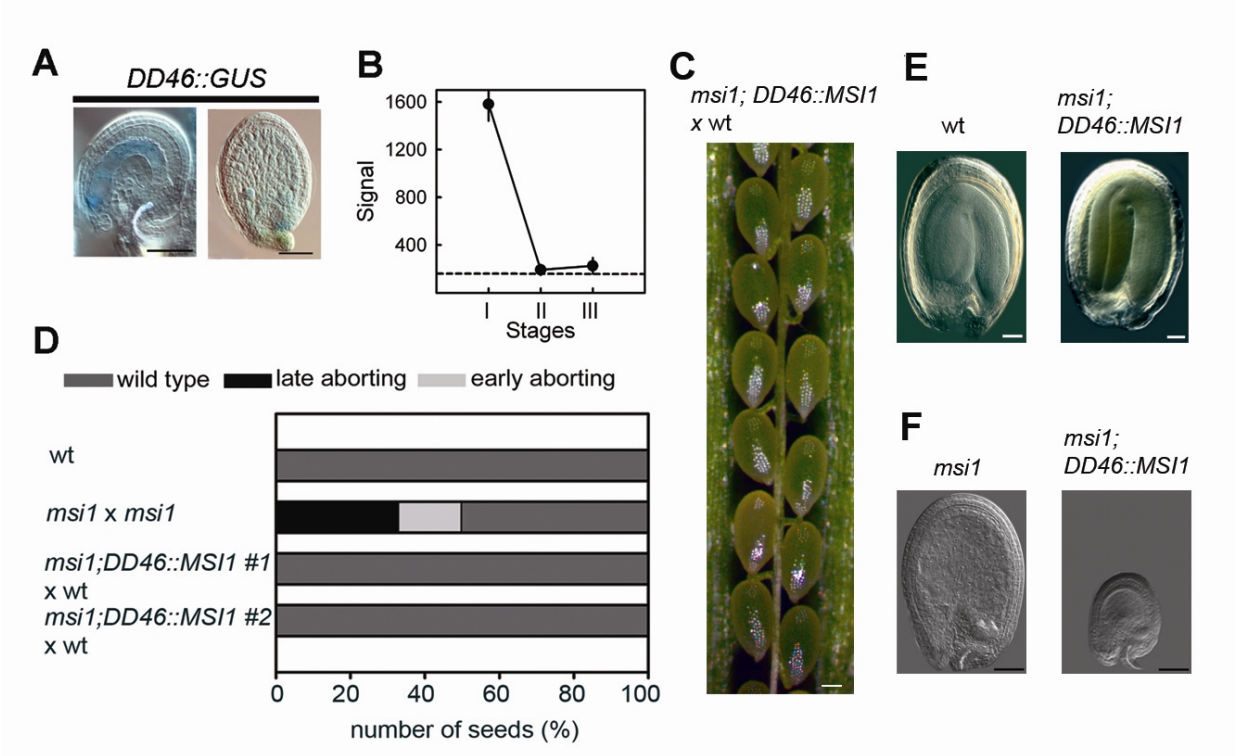

Fig. 4.6 Expression of *MSI1* before and shortly after fertilization can rescue the female gametophytic *msi1* mutant phenotype. (**A**) *DD46::GUS* expression can be detected in the female gametophyte (left panel). Residual expression of *DD46::GUS* in seeds at 2 DAP (right panel). (**B**) Expression of *DD46* on microarrays. *DD46* is expressed before fertilization (stage I) and is reduced to baseline levels after fertilization (stage II) and during seed development (stage III) (data from Hennig et al., 2004). Dashed line represents baseline. (**C**) *msi1/MSI1;DD46::MSI1* plants pollinated with wild-type pollen do not form aborting seeds. (**D**) Quantification of seed abortion observed after crosses of wild-type (wt) x wt (n=269), *msi/MSI1* x wt (n=487), *msi1/MSI1;DD46::MSI1 #1* x wt (n=369), *msi1/MSI1;DD46::MSI1 #2* x wt (n=475). (**E**) Cleared seeds of *msi1/MSI1;DD46::MSI1* pollinated with wild-type pollen. Seeds of this cross (right panel) are indistinguishable from wild-type seeds (left panel). (**F**) *msi1/MSI1;DD46::MSI1* plants do not form endosperm without fertilization (right panel). Autonomous endosperm development in *msi1* mutants at a similar time point (left panel). Scale bars: 50 µM in A, E, F, 200 µM in C.

IV.2.5 Expression of *FIS2* after fertilization cannot rescue the *fis2* female gametophytic defect

The previously collected data indicated that early paternal expression of *MSI1* after fertilization could not rescue the seed abortion caused by the maternally inherited *msi1* allele (Köhler et al., 2003b). This suggests that lack of function of the FIS complex in the female gametophyte causes seed abortion. However, *MSI1* is part of different complexes (Hennig, 2005). Therefore, to unequivocally address the question whether the FIS complex is needed before fertilization, I tested the temporal requirement of the specific FIS subunit FIS2 (Chaudhury et al., 1997; Luo et al., 1999).

I tested whether early paternal expression of *FIS2* under control of the *PHERES1* promoter (referred to as *PHE1::FIS2*) could rescue *fis2* mutant seeds. A heterozygous *fis2* mutant plant has 50% of mutant ovules and after fertilization, all the mutant ovules give rises to seeds that abort containing heart stage embryos (Fig 4.7 A, B and C). The construct *PHE::FIS2* was introduced into *fis2/FIS2* plants. Among twelve *fis2/FIS2* plants containing the *PHE::FIS2* construct, no plant with less than 50% seed abortion was identified (data not shown). Similarly, pollination of *fis2* plants with pollen of two independent *PHE1::FIS2* transgenic lines in a *fis2* background did not rescue mutant seeds (Fig 4.7 C), indicating that the *fis2* defect could not be rescued paternally. I analyzed seeds of this cross by clearing and found no significant change of seed development compared to seeds developing on self pollinated *fis2* plants (Fig 4.7 B).

To unequivocally verify whether early paternal *FIS2* expression can rescue the *fis2* mutant phenotype, I tested transmission of the maternal *fis2* allele after pollination of *fis2* plants with pollen of *PHE1::FIS2* plants. Therefore, I crossed *fis2* plants with pollen of two independent *PHE1::FIS2* transgenic lines in a wild-type background. As the *fis2* mutant allele I used was not associated with a resistance gene, I limited the investigation on 48 F1 plants that were analyzed for seed abortion. Among the tested F1 progeny none showed seed abortion, indicating that no *fis2* allele was maternally transmitted. Therefore, I conclude that early paternal *FIS2* expression is not sufficient to rescue the female gametophytic defect of *fis2* mutant seeds.

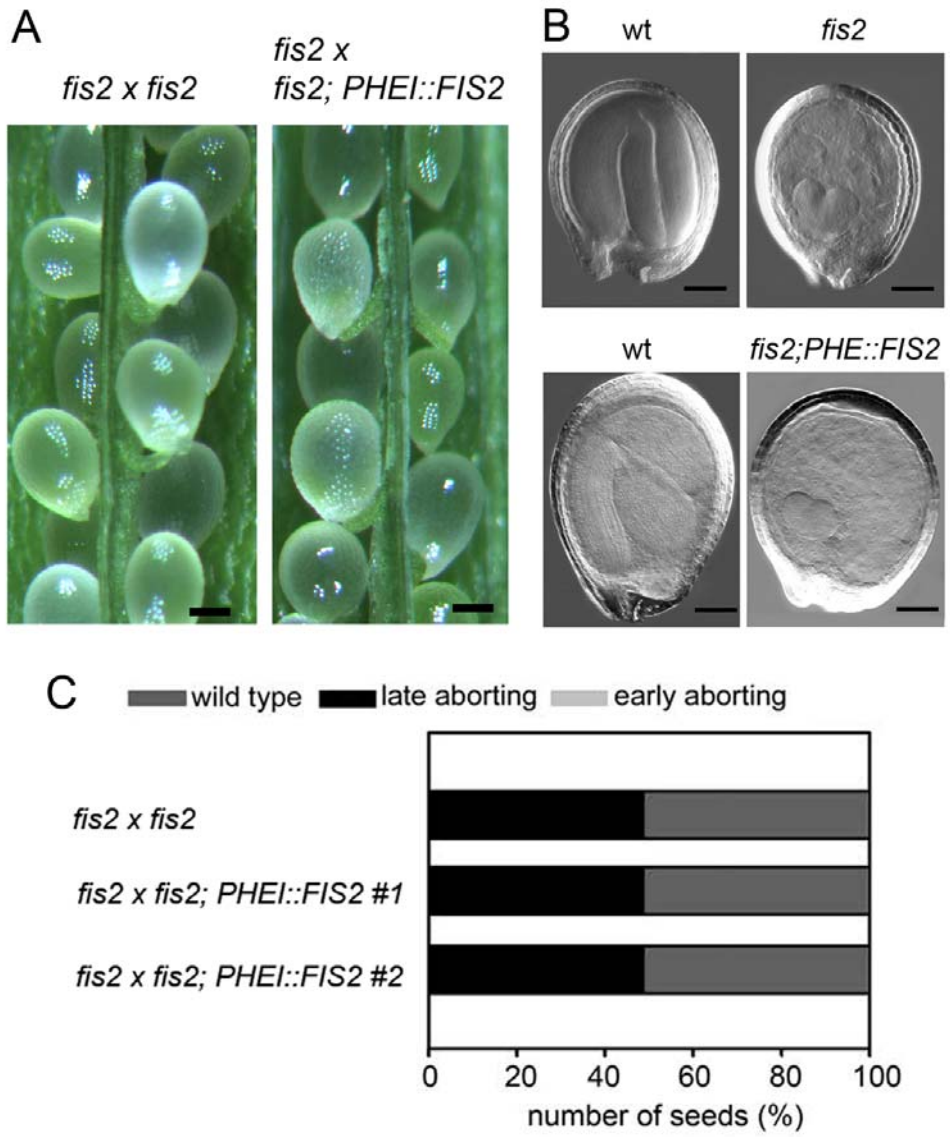

Fig. 4.7 Early paternal *FIS2* expression does no rescue the *fis2* mutant phenotype. (**A**) *fis2* mutants pollinated with *fis2/FIS2* pollen (left panel) or *fis2/FIS2;PHE1::FIS2* pollen (right panel) have 50% aborted seeds. (**B**) Cleared seeds derived from the same silique of a *fis2* mutant pollinated with *fis2* pollen (upper panels) or with *fis2;PHE1::FIS2* pollen (lower panels). (**C**) Quantification of seed abortion observed after crosses of *fis2/FIS2* x *fis2/FIS2* (n=1027) and *fis2/FIS2* x *fis2/FIS2;PHE1::FIS2* #1 (n=694), *fis2/FIS2* x *fis2/FIS2;PHE1::FIS2* #2 (n=667), Scale bars: 200 µM in B, 100 µM in C.

CHAPTER V DISCUSSION

V.1 Modifier mutants *mod16* and *mod41* suppress the *msi1* fis phenotype

During this study, two suppressor mutants of the *msi1* autonomous seed development phenotype were identified (Chapter III, fig 3.3): *modifier16* and *modifier41* (*mod16* and *mod41*). The aim of this study was to uncover genes regulating endosperm growth before fertilization and potential target genes of the FIS PcG complex. It is well possible that the same genes are required for endosperm development before and after fertilization, causing sterility of the suppressor mutants. Therefore, another goal of this thesis was to dissect the differential developmental mechanism of the autonomously growing seed versus the fertilization induced seed.

The mutations *mod16* and *mod41* do not completely inhibit autonomous endosperm development, but severely restricted it to a few mitotic cycles (Chapter III, fig 3.4). Furthermore, parthenogenetic embryo development is suppressed by both *mod* mutations, suggesting that *MODIFIERS* are necessary for the autonomous growth of both parthenogenetic embryo and endosperm.

Both *mod* loci are physically linked to the *msi1* locus, but are not allelic (Chapter III. 5.3). This is likely a consequence of the fact that only a few autonomous seeds are able to trigger silique elongation. Therefore, it is likely that other suppressors of the *msi1* fis phenotype that are physically unlinked from the *msi1* locus were not identified during the screening procedure and we preferentially selected for *msi1*-linked mutations, where almost all ovules have the *msi1, mod* genotype.

V.2 Modifier mutants *mod16* and *mod41* affect *msi1* seed development and are embryo lethal

In contrast to the suppressive effect of the *mod* mutations on the fis phenotype of the *msi1* mutant, *mod* mutations strongly enhanced the seed abortion phenotype of heterozygous *msi1* seeds. Indeed, whereas *msi1/MSI1* seeds arrest development at late heart stage, *msi1/MSI1, mod/mod* seeds arrested development as ovule-sized structures containing overproliferated globular stage embryos with very few endosperm nuclei (Chapter III, Fig 3.6 and Fig 3.7). Thus, the *mod* mutations synergistically interact with the *msi1* mutation after fertilization, suggesting that *MSI1* and *MOD* genes act in parallel pathways regulating endosperm development. Surprisingly, no genetic enhancement was observed in the double mutant *fis2; mod* (Chapter III, fig 3.9). Given that *fis* and *mod* mutants affect endosperm development, *mod* and *fis2* mutants should act in the same pathway to regulate endosperm growth. This prediction is in clear contradiction to the assumption that *MSI1* and *MOD* act in parallel pathways. Therefore, an alternative hypothesis to explain the synergism of the *msi1*, *mod* phenotype predicts that *fis* mutants are not functional null mutants. It is possible that there is sporophytic maternal carryover of *FIS* RNA present in *fis* mutant gametophytes, similar to the situation in the female zygotes of *Drosophila* and mammals. Therefore, if MOD is part of the FIS complex, lack of MOD could synergistically enhance the *fis* mutant phenotype. This phenotype would be significantly more pronounced in the *msi1* mutant than in the *fis2* mutant, as FIS2 might act redundantly with other genes like *EMF2* and *VRN2*. The fact that the penetrance of the fis phenotype in the *fis2* mutant is significantly weaker compared to the *msi1* mutant, supports this assumption. Future investigation will focus on the phenotypic characterization of the *fis2; mod* double mutant seeds to clarify the genetic interaction between *mod* and *fis2* mutations.

Surprisingly, more than half of the aborted seeds of *msi1, mod* plants aborted very early as ovule-sized structures (Chapter III, Fig 3.6 and Fig 3.7). This number is higher than expected, as heterozygous *mod/MOD* seeds developed normally (Chapter III, Fig 3.8). If we assume that the *mod* mutation suppresses the paternal *msi1* transmission defect, maximally 25% of total seeds (or 50% of *msi1* seeds) are expected to have the genotype *mod/mod* with

msi1 being mainly homozygous. The paternal transmission rate of *mod* upon wild-type or *mod* ovules follows, however, the transmission rate of the linked *msi1* allele (Chapter III, Table 3.3 before fertilization and Fig 3.8). One possible explanation for the higher number of ovule-sized structures formed in self fertilized *msi1, mod* plants is that *msi1, mod* pollen has a growth advantage over wild-type pollen and out-competes wild-type pollen for fertilization of *msi1,mod* ovules. This hypothesis predicts a higher paternal transmission rate of the *mod* mutation that will be tested in future investigations.

Whereas *msi1, mod/mod* seeds arrested development very early only after few divisions of the endosperm, initial development of the endosperm of *mod/mod* seeds without the *msi1* mutation was normal (Chapter III Fig 3.8). Whether the endosperm can cellularize and whether endosperm domains are properly established, awaits further investigations. However, based on the finding that *mod* mutations enhance the endosperm defect of the *msi1* mutant, it is conceivable that the primary defect caused by the *mod* mutation affects the endosperm and early embryo arrest in *mod* mutant seeds is a consequence of a failure in endosperm development. This hypothesis is supported by results from this study as well as by data from Novack and colleagues (2007), demonstrating an important role of the endosperm to sustain normal embryo development.

In conclusion, the two identified *MOD* loci play a role in endosperm development and suppress the *msi1* fis phenotype and enhance the *msi1* seed abortion phenotype. Both phenotypes are consistent with a predicted function for *MOD* genes to promote endosperm growth and/or differentiation.

V.3 MSI1 has sporophytic zygotic functions

The *msi1* mutant shares the parent-of-origin-dependent seed abortion phenotype with other mutants of the *fis* mutant class. Every seed inheriting a maternal *fis* allele aborts, regardless of the paternal contribution. However, in contrast to other *fis* mutants, *msi1* mutant seeds form two phenotypically distinguishable classes. In this study I showed that

the phenotype of early seed abortion is coupled to homozygous *msi1*/*msi1* seeds (Chapter IV, Fig 4.1). In contrast, seeds aborting with a *fis*-like phenotype are heterozygous *msi1/MSI1* mutant seeds derived from an *msi1* mutant female gametophyte (Chapter IV, Fig 4.1). Besides being a member of the FIS complex and related PRC2-like complexes, MSI1 is potentially part of several other chromatin modifying complexes (Hennig et al., 2005). A central role of MSI1 in plant development is supported by the observation that reduced MSI1 levels in *MSI1* cosuppression plants affect many aspects of sporophytic plant development (Hennig et al., 2003). Consistent with this idea is the observation that transmission of the *msi1* mutant allele through the male gametophyte is significantly reduced, suggesting that lack of *MSI1* function also impairs male gametophyte development.

In pollen of *FIE* cosuppression plants, the paternally silenced *MEA* allele becomes reactivated (Jullien et al., 2006), suggesting that FIE is necessary for repression of *MEA* and other paternally silenced genes in pollen. It is conceivable that this repression requires a functional PRC2-like complex and MSI1 is part of this complex. Therefore, one possible function of *MSI1* during pollen development could be the repression of paternally imprinted genes like *MEA* and *FIS2*. Alternatively, *MSI1* could be needed for activity of CAF-1 during pollen development. Future studies are needed to clarify which molecular function of *MSI1* is needed during pollen development. Such functions could include participation in PRC2-like complexes, in CAF-1 or in other, uncharacterized complexes.

V.4 *MSI1* is biallelically expressed in the embryo and the endosperm

It has been hypothesized that the maternal effect of *fis* mutants is caused by lack of expression of paternal *FIS* alleles; and indeed the paternal alleles of *MEA* and *FIS2* are imprinted (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000; Jullien et al., 2006). However, my results demonstrate that this does not apply to all *fis* mutants. I show that *MSI1* is not paternally imprinted, but clearly biallelically expressed in embryo and endosperm (Chapter IV, Fig 4.2). Transcripts of the paternal *MSI1* allele accumulate only

with a delay to the maternal *MSI1* allele's transcripts. However, timing of paternal *MSI1* expression is comparable to a large number of genes investigated thus far (Vielle-Calzada et al., 2000). Thus, *MSI1* is not paternally imprinted. Expression of the paternal allele of the *FIS* class gene *FIE* also occurs around two to three days after pollination, and it has been discussed that the parent-of-origin effect on seed development in *fie* and *mea* mutants is caused by different mechanisms (Yadegari et al., 2000). However, it has not been investigated, whether delayed expression of the paternal *FIE* allele is responsible for the parent-of-origin effect of *fie* mutants.

I tested whether delayed expression of the paternal *MSI1* allele is responsible for the *msi1* mutant phenotype by expressing *MSI1* under control of a promoter that is paternally active immediately after fertilization. As early paternal *MSI1* expression did not rescue seed development (Chapter IV, Fig 4.5), I concluded that *MSI1* functions in the female gametophyte and establishes gene expression patterns that were required for development of the seed after fertilization. Interestingly, I also did not observe rescue of seed development when expressing the *FIS2* gene under control of the *PHE1* promoter (Chapter IV, Fig 4.7). In contrast to the biallelically expressed *MSI1* gene, the paternal allele of *FIS2* is not active in the endosperm, thus *FIS2* is likely a paternally expressed imprinted gene (Luo et al., 2000; Jullien et al., 2006). Still, early paternal expression is not sufficient to rescue the *fis2* mutant phenotype. Therefore, I conclude that genomic imprinting of the *FIS* genes is not causing the parent-of-origin effect on seed development. Instead, the parent-of-origin-effect of *fis* mutants is caused by a lack of expression of the *FIS* genes in the female gametophyte at least for *MSI1* and *FIS2.*

V.5 *MSI1* activity in the female gametophyte affects seed development

PRC2-like complexes have histone methyltransferase activity, and this activity of the FIS2 complex appears necessary for normal seed development (Gehring et al., 2006; Makarevich et al., 2006). It is likely that genes marked by histone methylation in the female gametophyte need to be kept silent after fertilization. Indeed, the FIS target gene *PHE1* is methylated in the female gametophyte before fertilization and lack of *FIS* function causes strong overexpression of *PHE1* after fertilization (Köhler et al., 2003b, Makarevich et al., 2006). Thus, the FIS complex establishes epigenetic modification on its target genes that cause stable silencing during subsequent cell divisions. This function is consistent with the proposed role of PRC2 complexes in animals to stably maintain established repressive transcriptional states (Bantignies et al., 2006). A similar function has been assigned to the PRC2-like complex containing the FIS2 homolog VERNALIZATION2 (VRN2) (Gendall et al., 2001). *VRN2* is required for the vernalization dependent stable repression of the *FLOWERING LOCUS C* (*FLC*) gene. In *vrn2* mutants the initial repression of *FLC* after vernalization is not impaired, however, *FLC* repression is not stably maintained during subsequent periods of warm conditions (Gendall et al., 2001).

V.6 Function of the FIS complex after fertilization

All *FIS* genes are also expressed after fertilization in the endosperm (Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Köhler et al., 2003b) suggesting that the FIS complex has additional functions after fertilization, and it has been demonstrated that the FIS complex is necessary for suppression of the paternal *MEA* allele in the endosperm (Gehring et al., 2006; Jullien et al., 2006). Comparing the rescuing effects of the *PHE1::MSI1* and the *DD46::MSI1* expression upon the seed maternally inheriting the *msi1*allele, we found that expression of *MSI1* before fertilization in *msi1* mutant gametophytes is necessary to restore wild-type seed development (Chapter IV Fig 4.6). As

DD46 is also active until two to three days after pollination, I could not address the question whether expression of *MSI1* in the female gametophyte is also sufficient to rescue the *msi1* female gametophytic defect.

Seeds lacking a functional FIS complex have strongly overproliferated chalazal endosperm domains similar to seeds resulting from interploidy crosses of diploid maternal plants pollinated with pollen from tetraploid plants (Scott et al., 1998). Therefore, it has been hypothesized that the FIS complex regulates genomic imprinting and represses transcription of loci in the maternally derived genome that are normally expressed only when paternally contributed (Spielman et al., 2001). Consistent with this prediction is the expression of the FIS target gene *PHE1* that is maternally repressed and paternally active (Köhler et al., 2005). Furthermore, pollination of *fis* mutants *mea*, *fie* and *fis2* with pollen of the *cdka;1* mutant that only forms one generative cell causes the formation of viable seeds containing a normal zygotic embryo and homodiploid endosperm. Thus, bypassing the paternal contribution can rescue *fis* mutant seeds, providing strong support for this hypothesis (Nowack et al., 2007). Therefore, it is likely that the FIS complex-mediated genomic imprinting of *PHE1* and other still unknown genes is established in the female gametophyte and is maintained by the FIS complex after fertilization.

V.7 Embryo patterning is not affected in *fis* mutant embryos

After fertilization, the FIS complex mainly acts in the endosperm, and *fis* mutants including *msi1* have defects in endosperm development (Grossniklaus et al., 1998; Kiyosue et al., 1999; Köhler et al., 2003b; Guitton et al., 2004). Abortion of *fis* seeds is preceded by an arrest in embryo development, and I hypothesized that defects established in *fis* mutant gametophytes affect embryo pattern formation and cause developmental arrest of heterozygous *fis* mutant embryos. However, all markers of early embryo development and cellular differentiation tested in this study were expressed with similar patterns in wild-type and *fis* mutant embryos (Chapter IV, Fig 4.3). I did not observe changes in expression of markers genes for auxin distribution, shoot and root apical meristem regions, provascular

tissues and suspensor identity, indicating that there are no major defects in the establishment of the apical-basal axis as well as radial pattern formation. Therefore, I hypothesize that the female gametophytic defect caused by the *fis* mutations does not directly impact on embryo pattern formation and that embryo arrest occurs by as yet undefined mechanisms.

There are two possible explanations for the developmental arrest of heterozygous *fis* mutant embryos, (i) the arrest occurs after pattern formation by an inherent defect of the embryo, or (ii) embryo arrest is caused by an external defect, that is in the endosperm. Several observations favor the second hypothesis. Embryo arrest of *fis* mutant seeds occurs at late heart stage. Whereas the endosperm of wild-type seeds starts to cellularize at this stage and nuclei proliferation ceases (Brown et al., 1999; Boisnard-Lorig et al., 2001), endosperm of *fis* mutants does not undergo cellularization and instead continues to divide (Kiyosue et al., 1999). The endosperm of many dicotyledonous species such as *Arabidopsis* is non-persistent and considered as a transient medium supporting embryonic morphogenesis and early maturation by controlling the flux of nutrients delivered from the maternal plant to the developing embryo (Lopes and Larkins, 1993). The embryo is surrounded by the endosperm and both, embryo and endosperm need to coordinate their development in order to produce viable seeds. Hyperproliferation of the endosperm caused by an increased paternal dosage also inhibits embryo growth, implicating that increased proliferation of the endosperm is detrimental for embryo development (Scott et al., 1998). Conversely, bypassing the paternal contribution in *fis; cdka;1* double mutant seeds restores almost wild-type like embryo development (Nowack et al., 2007). It is conceivable that prolonged proliferation of the endosperm deprives the embryo from nutrients, or alternatively, that the endosperm does not reach the appropriate developmental stage to deliver nutrients to the developing embryo. Therefore, I suggest that lack of the FIS complex in the female gametophyte causes abnormal gene expression patterns in the central cell that persist after fertilization and produce defects in the endosperm that ultimately trigger arrest of embryo development and seed abortion.

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CURICULUM VITAE

Olivier Jean Michel LEROY
Born February 10th 1976,
French Nationality

Current Adress
Ellen Widmann Weg 4
8050 Oerlikon
Switzerland
tel: 078 624 18 36
olivier_leroy76@yahoo.fr

PHD STUDENT IN LIFE SCIENCE

sp. plant developmental biology

FORMATION AND RESSEARCH ACTIVITIES

2004 — present **PhD in plant developmental biology**

Role of MSI1 in reproductive development of flowering plants

3 years PhD in Laboratory of Prof Köhler, ETHZ, Institute of Plant Biology, Zurich

2004 **Training period in plant technologies**

Localization and effect of a exogenous peptide involve in apoptosis of *Arabidopsis thaliana*

3 months Training period in Laboratory of Dr BEVEN, UMR6022 of CNRS, UTC, Compiègne

Research assistant (Switzerland-France collaboration)

2003

Specific promoter interactions of homologous sigma S and sigma 70 subunit of RNA polymerase during initiation of transcription

1 month, Research Assistant in the Laboratory of Dr. LANDINI, EAWAG, Dübendorf

6 months, Research Assistant in the Laboratory of Dr. KOLB, Pasteur Institute, Paris

Exchange student

2001 –2003 **1 & 1'-Micromovements of RNA polymerase during abortive initiation and elongation phase of transcription.**
2-*In vivo* sigma release off the RNA polymerase.

2 years Invited Scientist in the Laboratory of Prof Richard EBRIGHT, Waksman Institute, Rutgers University, USA

Master of fundamental microbiology (Paris VII)

1999 –2000 ***In vivo* and *in vitro* studies of sigma S region 2.5 mutants of the RNA polymerase of *Escherichia coli***

1 year Training period in the Laboratory of Dr KOLB, Pasteur Institute, Paris

6 months Training period in the Laboratory of Dr DUJON, Pasteur Institute, Paris

REFERENCES

ETHZ, Institute of Plant Sciences Universitätstrasse 2 CH-8092 Zurich, Switzerland
Prof. Dr. Claudia Köhler, Laboratory of Plant Developmental Biology
claudia.koehler@ipw.biol.ethz.ch

Univeristé de Bordeaux, INRA, UMR 1090, F-33883 Villenave d'Ornon Cedex
Dr. Laure BEVEN, Unité de Génomique Diversité pouvoir Pathogene
laure.beven@bordeaux.inra.fr

Univeristy of Milan, Via Celoria 26, 20100 Milan, Italy
Dr. Paolo LANDINI, Department of Biomolecular Sciences and Biotechnology
Paulo.Landini@unimi.it

Waksman Institut, Rutgers University, 190 Frelinghuysen road 08854 Piscataway NJ, USA
Prof. Dr. Richard EBRIGHT, Chemistry department
ebright@waksman.rutgers.edu

Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
Prof. Dr. Bernard DUJON, Unité de genetique moléculaire des levures
bdujon@pasteur.fr

Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
Dr. Annie KOLB, Unité des régulations transcriptionnelles,
akolb@pasteur.fr

LANGUAGES

French and English: fluent; German: in learning process

PUBLICATIONS

Olivier Leroy, Lars Hennig, Helmut Breuninger, Thomas Laux, Claudia Köhler.
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